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Articles over two printed pages in length (i. e. about 800 words) must be preceded by a brief summary (100–400 words) which must be complete in itself and contain all the important facts brought out in the paper. When possible the abstract should summarize the new methods in such form that a reader can follow the directions without consulting the body of the paper.

All staining formulae must be definite and accurate. The batch or certification number of the stains actually used by the author in such formulae should be included for reference purposes. In case of uncertified stains, not designated by any batch number, one should give as much information as available, i. e. name of manufacturer, approximate date of purchase, etc.

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STAIN TECHNOLOGY

VOLUME 25 APRIL, 1950 NUMBER 2

SPECTROPHOTOMETRIC CHARACTERISTICS AND ASSAY OF BIOLOGICAL STAINS

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ABSTRACT.—**I. The chemical and spectrophotometric methods adopted in the Stain Commission laboratories for the certification of stains are described. Spectrophotometric examination is by the Beckman spectrophotometer in which the color density at the absorption maximum is determined as well as a ratio of color densities designed to detect abnormal symmetry of the absorption curve. A somewhat modified titanous chloride assay is described; ceric sulfate is used to determine the normality of the unstable titanous chloride, and the oxidant solution, which is stable, is standardized against Bureau of Standards arsenious oxide. The general approach used for re-study of the stains by these methods and the criteria necessary for adoption of the spectrophotometric method of assay are described.**

II. This report gives the details of the spectrophotometric examination and assay of the individual stains in the thiazin group, namely methylene blue, thionin, methylene violet, toluidine blue O, and azures A, B, and C. The spectrophotometric method of assay was adopted for the first three named; the others showed too large a variation among samples to permit adoption of this simpler type of assay. In the case of methylene violet it was necessary to do nitrogen analyses to relate purity of the dye and color density. Complete absorption curves for typical samples of each of the above stains are presented.

I. GENERAL METHODS

ELMER STOTZ and H. J. CONN

This is the first of a series of papers which will deal with the

spectrophotometric identification and methods of assay developed in these laboratories for the certification of the commonly used biological stains. Prior to the work reported, a visual spectrophotometer was employed and a simple ratio of color densities at two selected wavelengths was used to express the color characteristics. With the availability of the Beckman photoelectric spectrophotometer, it became possible to characterize the stains by complete absorption spectra and by exact location of the absorption maximum. The same type of ratio was temporarily retained, which along with the position of the absorption peak, served for the comparison of different samples of the same stain. Some of these data have been published (Conn and Stotz, 1946; Darrow et al., 1949).

At the outset of the work reported in this and succeeding papers of the series, it was optimistically planned that the assay of all the stains might be accomplished by the spectrophotometric method. This proved possible with those stains whose manufacture is apparently sufficiently standardized to result in successive samples with quite identical spectral characteristics. The color density at the maximum absorption of such stains was easily related to the dye content as determined by other methods. In the case of stains in which various samples showed considerable variation in the position of the absorption peak, it was impossible to establish the spectrophotometric assay as a satisfactory substitute for the titanous chloride or color-acid-precipitation methods. During the course of these investigations it was also found necessary to re-evaluate the "standard" titanous chloride method and some changes have been made.

In the following section some of the general methods of chemical examination of stains for certification are described, after which more specific details concerning each dye of the thiazin series will be given.

Spectral Characteristics

A 50 mg. sample of the homogenous (powdered if necessary) stain is weighed to the nearest 0.5 mg. on a Roller-Smith torsion balance. The sample is transferred quantitatively to a 250 ml. volumetric flask and approximately 225 ml. of the appropriate solvent added. The flask is then vibrated on a Boerner shaker[1] for 20 minutes to insure complete solution of the dye. The solution is made up to the mark with solvent, thoroughly mixed, and an appropriate aliquot taken for further dilution to provide a satisfactory dye concentration for spectrophotometric work.

Maximum accuracy of weighing and insured complete solution of the dye is of course dispensable if the spectrophotometric assay is not employed.

Matched Corex cells of 1 cm. light path are employed in the Beckman DU

[1]Arthur H. Thomas Co.

spectrophotometer, the reference cell containing the same solvent as the dye solution. Spectrophotometric readings are taken at least every 5 mμ and usually at smaller intervals in the area of the absorption maximum. An actual reading at the exact peak is of course desirable for spectrophotometric assay.

The older method of expressing the shape of the curve by the ratio of color densities at two predetermined wavelengths loses its significance if the exact peak is known, since any differences in the position of the peak necessarily cause a change in the ratio. More elaborate methods have been used to express the shape of the curve independently of the peak position (Lillie and Roe, 1942) but we have chosen the simple ratio of the color densities at minus and plus 15 mμ of the peak wavelength found with the given sample. These densities can be estimated from the plotted spectrum or an absolute figure actually measured after location of the peak.

Titanous Chloride Assay

A study of the titanous chloride standardization, use of the solution, and the dye titration itself led to various modifications. Titanous chloride solutions are standardized against ceric sulfate, which in turn is standardized against arsenious oxide. Both titrations employ ferrous-o-phenanthroline indicator which gives sharp endpoints.

Sodium Arsenite—Dissolve 4.947 g. of U. S. Bureau of Standards arsenious oxide (As_2O_3) in a 600 ml. beaker containing 200 ml. of distilled water and 5 g. of sodium hydroxide pellets. After complete solution add 13 ml. of concentrated hydrochloric acid, mix, and then add 10 g. of sodium bicarbonate slowly to prevent loss by spray. Transfer the solution quantitatively to a 1-liter volumetric flask, dilute to the mark, and mix. The resulting solution is 0.1 *N*.

Ceric Sulfate—Weigh 66.5 g. of anhydrous ceric sulfate [$Ce(SO_4)_2$] into a 600 ml. beaker and add 28 ml. of concentrated sulfuric acid. Add cautiously about 30 ml. of distilled water with stirring. Heat the solution, stir, and add successive portions of water until the salt is completely dissolved. Cool, transfer the solution to a 1-liter volumetric flask and dilute to the mark. This approximately 0.1 *N* ceric sulfate solution is completely stable.

Iodine Monochloride Catalyst—Dissolve 10 g. of potassium iodide (KI) and 6.74 g. of potassium iodate (KIO_3) in 90 ml. of distilled water. Add 90 ml. of concentrated hydrochloric acid and mix. This solution is 0.5 molar in iodine monochloride. It should be stored in the dark.

Standardization of Ceric Sulfate—Pipette exactly 25.0 ml. of 0.1 *N* sodium arsenite into a 200 ml. Erlenmeyer flask, add 20 ml. of concentrated hydrochloric acid, and 2.5 ml. of 0.005 *M* iodine chloride (dilute above stock). Dilute the mixture to about 100 ml., add 1 drop of ferrous-o-phenanthroline indicator[2], and

[2]"Ortho-Phenanthroline Ferrous Complex (Ferroin) 0.025 *M*". Purchased from the G. Frederick Smith Chemical Co., Columbus, Ohio.

titrate with the ceric sulfate until the orange color of the indicator returns only slowly after dropwise addition of the ceric solution. At this stage warm the solution to 50° C., add another drop of indicator, and continue the titration dropwise until a single drop produces a light green solution with no return of the orange color for at least one minute. The endpoint is sharp and reproducible. The normality of the ceric sulfate solution is calculated as follows:

$$\text{N ceric} = \frac{25 \times 0.1}{\text{ml. ceric}}$$

Titanous Chloride Solution—The stock solution is 20% titanous chloride stabilized with hydrochloric acid[3]. An approximately 0.05 *N* solution is prepared by diluting 8.0–8.5 ml. of the stock solution to 200 ml. with distilled water. Storage experiments with this diluted titanium solution showed that it could be stored under mineral oil for a normal working day without change, and for 20 hours with approximately a 1–2% decrease in strength. In practice the solution was placed in a burette equipped with a side reservoir, and both surfaces of the solution were covered with ½ inch of mineral oil. A fresh solution was made daily and the solution standardized against ceric sulfate as follows: Exactly 5 ml. of standard ceric sulfate was placed in a 50 ml. Erlenmeyer flask, and 5 ml. of concentrated sulfuric acid added. After adding a drop of ferrous-o-phenanthroline indicator, the solution was titrated with the stored titanium solution. The initial yellow color of the solution passes through a green stage, then nearly colorless, and finally a single drop of Ti^{+++} produces the orange color of the reduced indicator. The titration is rapid and the endpoint exceedingly sharp.

Dye Titration with Titanous Chloride

Minor changes have been made in the dye titration procedure. Smaller amounts of dye are used and a more dilute titanous chloride solution is employed than has been recommended in "Biological Stains, 5th Ed." In the case of most dyes we have preferred a strong acetate buffer to maintain a more constant pH during the titration, although in some cases the tartrate or bitartrate buffer has been retained. We also prefer to use nitrogen gas washed through two titanium-chloride-safranin bottles to maintain more anaerobic conditions and minimize autoxidation of reduced dyes during the titration. Following is a general description of the titanous chloride assay as carried out in our laboratory. Specific variations will be noted under the individual dyes.

The titration is carried out in an ordinary "Mason" jar of about 350 ml. capacity and a 2½ inch diameter mouth. A tight-fitting rubber stopper contains five holes for the accommodation of a gas inlet, gas outlet, the burette extension tip, a thermometer and a ground glass sleeve for the shaft of a stirring propeller. The titration jar is mounted on an asbestos covered hot-plate. Water-pumped tank nitrogen is led through two successive gas-washing bottles[4] containing tita-

[3]LaMotte Chemical Products Co., Baltimore, Md.

[4]Fisher-Milligan Gas Washer Bottles.

nous chloride safranin. The latter solution is made by diluting 40 ml. of 20% titanous chloride and 40 ml. of concentrated hydrochloric acid to 500 ml. with distilled water. After filling the gas-washing bottles, approximately 10 mg. of safranin is added to each bottle. This mixture is replaced by fresh solution every two or three days depending on amount of use.

The weighed dye sample is dissolved in the appropriate solvent in the titration jar, and buffer added. The total volume of dye mixture is usually 200 ml. The solution is stirred, heated if necessary, and the nitrogen passed through the jar for at least fifteen minutes before the titration is started. Like most titrations, the first run locates the approximate endpoint so that a second trial can be rapid and the type of endpoint color change familiar.

The per cent dye is calculated by the general formula:

$$\text{per cent dye} = \frac{\text{ml. } TiCl_3 \text{ used} \times \text{normality of } TiCl_3 \times \text{mol. wt. of dye} \times 100}{\text{Wt. of sample} \times \text{No. of hydrogen equivalents} \times 1000}$$

General Approach to Examination of Individual Stains—Study of the individual stains by the general methods described consisted of measuring the spectra of a series of samples of recent origin and of satisfactory staining quality. Ratios of the color density at the peak (D–peak) to previously recorded assay values were then calculated and compared. If these ratios (Assay/D–peak) were reasonably constant, the chemical assays were carefully repeated, the ratios recalculated, and the average value used to serve as the necessary factor for spectrophotometric assay of the stain. If the ratios proved not to be reasonably constant, either the spectrophotometric data or the chemical assay was considered to be at fault. Spectrophotometric assay was obviously unsuitable when representative samples of the stain showed great variation in the position of the peak or other spectral characteristics. In such cases the chemical assay proved to be the method of choice.

In a few cases the titanous chloride assay was not entirely satisfactory, and it was difficult to establish a figure for per cent purity. In such cases nitrogen analyses were made and the per cent dye calculated on this basis. This figure in turn served to standardize the spectrophotometric assay of the dye.

It should be noted that the studies reported here and in succeeding papers of this series are designed specifically to provide a quick chemical and spectrophotometric evaluation of commercial stains as they are received for certification. As such, the standards set are based primarily on past commercial samples and their satisfactory use in staining procedure. From the strictly chemical standpoint it is recognized that "per cent dye" as determined by titanous chloride, spectrophotometry, or color base precipitation cannot adequately describe the true content of the dye specifically named. The complexity of the stains in the thiazin series is illus-

trated by the reports of R. D. Lillie (1944 a & b), in the case of the azures, methylene violet, and methylene blue. After completion of our primary objectives, it is planned to investigate the true composition of some of the commercial stains by the newer technics of counter-current distribution and partition chromatography.

II. THE THIAZINS

FRANCES KNAPP, ARTHUR J. EMERY, JR., and ELMER STOTZ

METHYLENE BLUE

This dye represents a typical case in which the position of the peak proved to be quite constant among different samples and a constant assay/D–peak could be established. The complete spectrum of a

TABLE 1. SPECTRAL AND ASSAY DATA FOR METHYLENE BLUE

Sample	Peak mμ	$\frac{P-15}{P+15}$	D-peak	Assay per cent*	$\frac{\text{Assay}}{\text{D-peak}}$
LA–14	665	1.45	0.659	85.1	129
LA–16	665	1.50	0.689	90.0	132
LA–17	665	1.67	0.680	88.1	130
CA–23	665	1.45	0.669	83.2	124
CA–24	665	1.45	0.689	86.1	125
CA–25	665	1.45	0.700	85.5	122
NA–26	665	1.58	0.656	84.8	129
NA–27	665	1.39	0.669	84.8	127
NA–28	665	1.35	0.687	89.3	130
NA–29	665	1.48	0.691	86.7	125
					Average = 127

*Titanous chloride.

typical methylene blue sample is included in Fig. 1, and representative spectral and assay data are recorded in Table 1. Beer's law of dilution was found to hold over the range of concentrations satisfactory for measurement according to the specified directions.

Spectral: 50 mg. methylene blue dissolved in 250 ml. distilled water; dilute 3 ml. of this solution to 200 ml. with distilled water. Read in Beckman. Absorption maximum 665 mμ; ratio $\frac{P-15}{P+15}$ from 1.35 to 1.70.

Assay: Spectrophotometric. Per cent dye = D–peak × 127. D–peak (color density at peak) measured on dye solution described under "Spectral".

THIONIN

It was also found possible to assay this stain spectrophotometrically. Beer's law of dilution was found to hold over the necessary

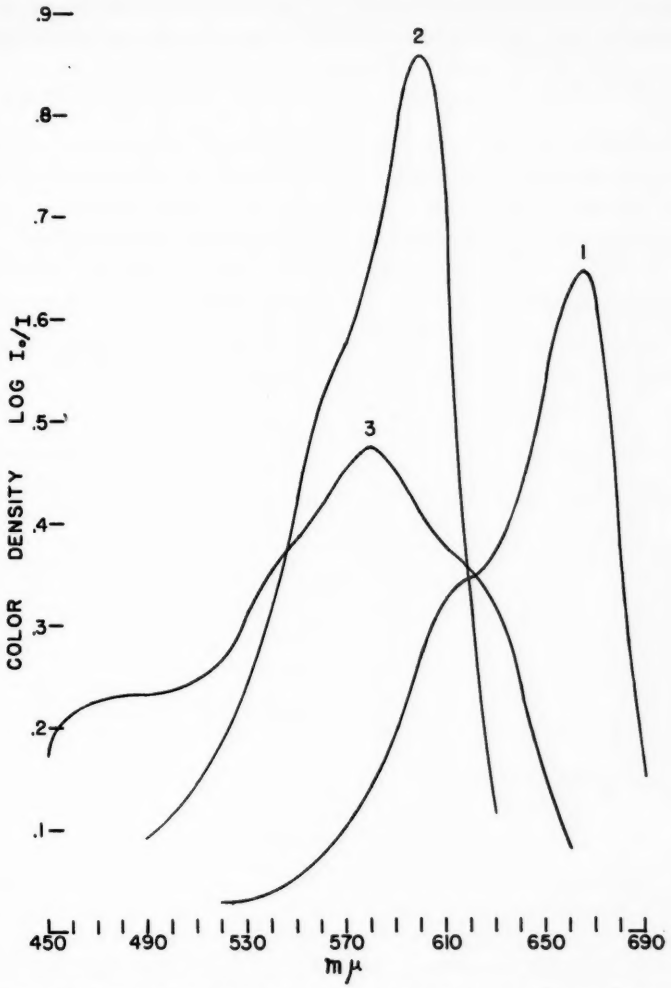

FIG. 1.—Spectral Curves of Methylene blue, Thionin, and Methylene violet. Curve 1, Methylene blue; 2, Thionin; 3, Methylene violet.

range. The complete spectrum is included in Figure 1, and representative spectral and assay data are recorded in Table 2. It may be noted from the latter table that three of the samples gave abnormal assay/D–peak values and that these showed low assays by the titanous chloride method. One of these samples (NT–15) was

rejected on the basis of staining performance, and the other two were not as satisfactory as the usual thionin samples. Since the position of the peak and the $\frac{P-15}{P+15}$ ratios of these abnormal samples were satisfactory, it would seem desirable to run both a spectrophotometric and titanous chloride assay on thionin samples with D–peak values below 0.8.

Spectral: 50 mg. thionin dissolved in 250 ml. distilled water; dilute 5 ml. of this solution to 200 ml. with distilled water. Read in Beckman. Absorption maximum 598–599 mμ; ratio $\frac{P-15}{P+15}$ from 1.15 to 1.35.

Assay: Spectrophotometric. Per cent dye = D–peak × 97. D–peak (color density at peak) measured on dye solution described under "Spectral".

TABLE 2. SPECTRAL AND ASSAY DATA FOR THIONIN

Sample	Peak mμ	$\frac{P-15}{P+15}$	D-peak	Assay per cent*	$\frac{\text{Assay}}{\text{D-peak}}$
NT–7	598	1.21	0.916	86.9	95
NT–8	598	1.20	0.938	92.6	99
NT–10	598	1.16	0.923	87.1	94
NT–11	598	1.25	0.921	86.6	94
NT–13	599	1.35	0.921	87.1	95
NT–14	599	1.32	0.821	85.0	104
				Average	= 97
NT–9	598	1.20	0.759	83.3	110
NT–12	598	1.21	0.786	84.9	108
NT–15	598	1.28	0.702	82.7	117

*Titanous chloride titration.

METHYLENE VIOLET

No methods had previously been devised for the chemical or spectrophotometric identification of this stain. Trials with the titanous chloride titration, even at different pH values, gave assays of greater than 100% dye, obviously due to over-reduction. Furthermore, spectra of the dye in 50% alcohol solutions were difficult to reproduce, apparently due to marked effects of acid and alkali on the color and to the low solubility of the dye. Spectral measurements proved to be reproducible when the dye was dissolved in acidified 50% alcohol. Beer's law of dilution held within the range of dye concentrations suitable for spectrophotometric work.

In order to use spectrophotometric assay, it became necessary to establish a relation between the color density of the dye and per cent

purity. For this purpose a sample (NLv–5) was recrystallized several times from hot dichloroethane. The products from the second and third recrystallization reached the same nitrogen content (Kjeldahl) of 9.53%. Based on the formula of methylene violet, this sample contained 87.03% anhydrous dye. The same sample was then subjected to careful spectrophotometric analysis and the color density at the peak (D–peak) determined. From these data, the necessary factor was obtained for spectrophotometric assay.

The spectrum of methylene violet is shown in Figure 1 and representative spectral and assay data recorded in Table 3.

Spectral: 25 mg. methylene violet dissolved in 125 ml. of 95% alcohol, add 10 ml. of 0.1 *N* HCl and dilute to 250 ml. with distilled water; dilute 10 ml. of this solution and 20 ml. of 0.1 *N* HCl to 200 ml. with 50% alcohol. Read in Beckman. Absorption maximum 579–580 mμ; ratio $\frac{P-15}{P+15}$ from 0.99 to 1.02.

TABLE 3. SPECTRAL AND ASSAY DATA FOR METHYLENE VIOLET

Sample	Peak mμ	$\frac{P-15}{P+15}$	D-peak	Assay per cent
NLv–5 (recrystallized)	579	1.00	0.480	87.03*
NLv–1	579	0.99	0.502	90.9**
NLv–2	579	1.02	0.488	88.3**
NLv–3	579	1.00	0.511	92.5**
NLv–5	580	1.01	0.344	62.3**

*Determined by nitrogen content.
**Calculated from D-peak values (per cent dye = D-peak × 181).

Assay: Spectrophotometric. Per cent dye = D–peak × 181. D–peak (color density at peak) measured on dye solution described under "Spectral".

TOLUIDINE BLUE O

Only recently submitted samples of this stain were subjected to spectrophotometric study because of the known instability of toluidine blue in storage. Even with this precaution the position of the absorption maximum varied considerably among samples. Furthermore this dye upon dilution did not follow Beer's law. Hence, toluidine blue was not suitable for spectrophotometric assay. The titanous chloride titration was found to be reproducible and the method of choice for assay.

A complete spectrum of one of the toluidine blue samples is included in Figure 2, and representative spectral and assay data are recorded in Table 4.

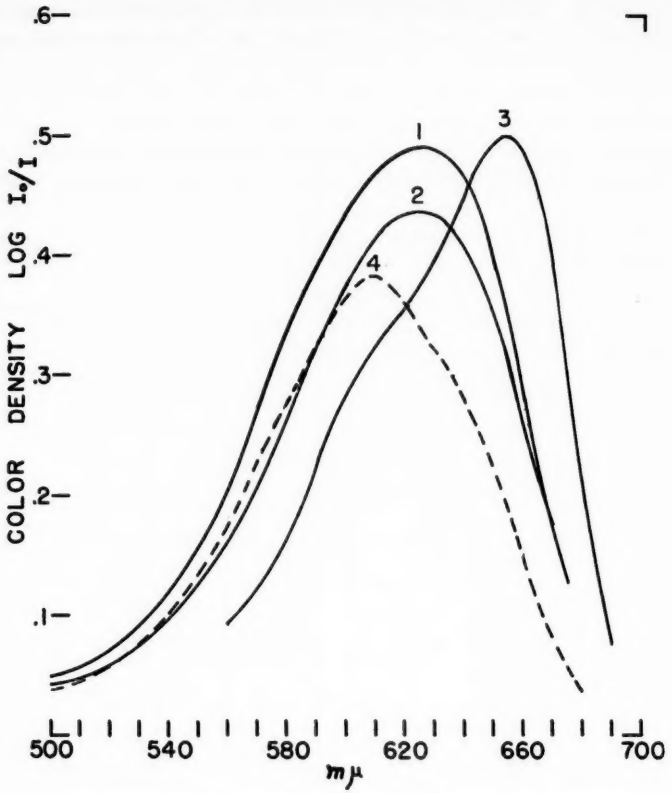

FIG. 2.—Spectral Curves of Toluidine blue, and Azures A, B, and C. Curve 1, Toluidine blue; 2, Azure A; 3, Azure B; 4, Azure C.

TABLE 4. SPECTRAL AND ASSAY DATA FOR TOLUIDINE BLUE O

Sample	Peak mμ	$\frac{P-15}{P+15}$	D-peak	Assay per cent*
NU-5	625	1.02	0.516	67.3
NU-6	620	1.01	0.470	65.6
NU-7	627.5	1.03	0.480	64.4
NU-8	632.5	1.09	0.546	70.0
NU-9	630	1.09	0.510	67.5
CU-3	627.5	1.03	0.470	66.1

*Titanous chloride titration.

Spectral: 50 mg. toluidine blue dissolved in 250 ml. of distilled water; dilute 5 ml. of this solution to 200 ml. with distilled water. Read in Beckman. Absorption maximum 620–632.5 mμ; ratio $\frac{P-15}{P+15}$ from 1.01 to 1.09.

Assay: Dissolve 100 mg. of toluidine blue in 175 ml. distilled water and add 25 ml. of pH 4.0 acetate buffer (6 volumes of 50% $NaAc{\cdot}3H_2O$ + 4 volumes glacial acetic acid). Heat to boiling and titrate with 0.05 *N* titanous chloride to a yellow endpoint.

Azure A, B, and C

These closely related dyes showed great variations in the position of their maximum absorption peaks. Some samples submitted are recognized mixtures, and others are clearly not single dyes. From a

Table 5. Spectral and Assay Data for Azure A, B, and C

Sample	Peak mμ	$\frac{P-15}{P+15}$	D-peak	Assay per cent*
		AZURE A		
NAz–11	625	1.01	0.523	90.0
NAz–12	625	1.05	0.493	87.7
NAz–13	622.5	0.94	0.473	78.0
NAz–14	625	1.01	0.451	92.5
LAz–5	630	1.01	0.303	60.5
LAz–6	632.5	0.99	0.296	53.0
LAz–10	632.5	0.98	0.289	58.9
		AZURE B		
NAb–1	652.5	0.94	0.460	82.3
NAb–2	654	1.12	0.500	75.5
LAb–1	647.5	1.05	0.490	81.1
LAb–2	647.5	1.11	0.478	82.2
		AZURE C		
NAc–1	609	0.98	0.319	63.9
NAc–2	607.5	1.03	0.407	81.7
NAc–3	607.5	1.00	0.383	77.6
NAc–5	610.5	0.98	0.482	92.6

*Titanous chloride titration.

considerable series of azures studied, certain samples have been selected for illustration which perhaps represent largely the component stated. Further work of separation and better spectral characterization of the individual azures are needed.

Because of the spectral variations among the azures, the spectrophotometric assay method was quite unsuitable. Fortunately, however, the titanous chloride titration is easily reproducible. Typical spectra for the azures are shown in Fig. 2, and spectral and assay data are recorded in Table 5.

Spectral: 50 mg. of azure sample dissolved in 250 ml. of distilled water; dilute 3 ml. of this solution to 200 ml. with distilled water. Read in Beckman.

Azure A—Absorption maximum 622.5–632.5 mμ; ratio $\frac{P - 15}{P + 15}$ from 0.94–1.05.

Azure B—Absorption maximum 647.5–654 mμ; ratio $\frac{P - 15}{P + 15}$ from 0.94–1.12.

Azure C—Absorption maximum 607.5–612.5 mμ; ratio $\frac{P - 15}{P + 15}$ from 0.98–1.03.

Assay: Dissolve 100 mg. of azure sample in 175 ml. distilled water and add 25 ml. of pH 4.0 acetate buffer (6 volumes of 50% $NaAc{\cdot}3H_2O$ + 4 volumes of glacial acetic acid). Heat to boiling and titrate with 0.05 *N* titanous chloride to a yellow endpoint.

REFERENCES

CONN, H. J., and STOTZ, E. 1946. Comparative absorption readings obtained with spectrophotometers of various types. Stain Techn., **21**, 49–54.

DARROW, M. A., KNAPP, F., STOTZ, E., and CONN, H. J. 1949. A comparison of American stains with recent German products. Stain Techn., **24**, 1–8.

LILLIE, R. D., and ROE, M. A. 1942. Studies on polychrome methylene blue. I. Eosinates, their spectra and staining capacity. Stain Techn., **17**, 57–64.

LILLIE, R. D. 1944a. The deterioration of Romanovsky stain solutions in various organic solvents. Supp. No. 178, Public Health Reports, p. 17.

LILLIE, R. D. 1944b. Factors influencing the Romanovsky staining of blood films and the role of methylene violet. J. Lab. and Clin. Med., **29**, 1181.

THE SELECTIVE STAINING OF MITOCHONDRIA

JOHN W. HARMAN, *Department of Pathology, Medical School, and Institute of Enzyme Research, University of Wisconsin, Madison, Wis.*

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ABSTRACT.—**A critical analysis of extant selective mitochondrial stains has elucidated certain empirical criteria for the adoption of dyes for trial. These criteria include a specific triphenylmethane structure for the dye, with sulfonation and the use of aniline and heat as adjuvants. By the application of these characteristics the dyes fast green FCF and light green SF yellowish were chosen for study and proved to be highly selective mitochondrial stains. They are applicable to both tissue slices and homogenate studies and permit examination of the internal structure of mitochondria.**

The separation of subcellular, enzymatically active granules from tissue homogenates has required the use of several cytologic technics for their identification. During the establishment of the identity of the specific units as mitochondria, the utilization of phase microscopy revealed an unexpected structural complexity in these bodies. The classical staining procedures of Altmann (1894) and Benda (1901) indicated no evidence of an internal structure within the mitochondria; preparations stained by either technic suggested no intrinsic variation in refractive density such as revealed by the phase microscope. But, because of the reliable uniformity with which this internal structure was disclosed by phase contrast in the numerous homogenates and teased preparations studied, the phenomenon was acceptable as genuine. The inability of the several staining procedures to render this manifest led to a closer analysis of such methods and finally to the selection of another dye and procedure by which the structure was suitably demonstrated.

Until the present, the methods of staining used most satisfactorily to demonstrate mitochondria have been either the original or some modification of the several procedures of Altmann (1894), Benda (1901) and Regaud (1909). The method of Regaud has been least specific; the other technics in their various forms are more precise, although both may be capricious and tedious, and require considerable manipulative experience. In unskilled hands the experimental errors may loom discouragingly and persistently great. A critical appraisal of these basic technics and of the modification of several workers including Bensley (1911), Kull (1913) and Volkonsky (1928) has elucidated the empirical criteria upon which these successful procedures depend. These characteristics are as follows:

1. The chemical constitution of the dyes is uniformly similar. The various specific dyes are structurally members of the triphenylmethane series and include acid fuchsin, crystal violet, acid violet and malachite green. Most of these dyes, except crystal violet, are sulfonated and distinctly acid.

2. The solvent for the dyes is a 10% (V/V[1]) solution of aniline oil in water into which a suitable quantity of the dye is weighed to form a satisfactory per cent (W/V[1]) solution.

3. Gentle heating is required to unite the dye firmly to the mitochondria, as compared with other cytologic units.

In conformity with these prescribed features the selection of dyes was limited to sulfonated diamino-triphenylmethane compounds. Of these, the most immediately available and satisfactorily standardized were light green SF yellowish (C.I. No. 670) and fast green FCF (Peterson, Conn and Melin, 1934), which are structurally similar. The fast green FCF was elected for extensive trial because of its suggested superiority to the other green dye. The decision to employ this dye was fortunate and speedily permitted the formulation of a procedure by which the study of mitochondrial detail was satisfactorily secured. The details of that investigation of mitochondrial structure will be reported elsewhere.

By direct experimentation a method of staining was elaborated, the several steps of which were so organized that the sharpest and clearest definition of mitochondria was assured, without simultaneously sacrificing any richness of histologic detail. The method was tried upon thin films of tissue homogenates fixed in a Coplin jar by vapor of 4% osmium tetroxide, and upon thin paraffin sections of tissues fixed by the technics of Altmann (1894) and Regaud (1909–10). The final procedure consisted of the following steps:

1. The slides (smears or deparaffined sections) are immersed in a 4% (W/V) solution of fast green FCF[2] in aniline water, 10% (V/V). The dye solution immediately prior to use is brought to a temperature of 62° C. in a Coplin jar containing 50 ml. of the dye, and the slides are exposed to this slowly cooling dye solution for 6 minutes.

2. After a rapid, thorough rinse in deionized water, the slides are transferred to a saturated aqueous picric acid for 10 minutes.

3. They are again rinsed with deionized water and placed in 1% phosphomolybdic acid for 1 minute, and then washed in deionized water for 1 minute.

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[1]The percentage composition of solutions is indicated on a weight to volume ratio by W/V and a volume to volume ratio by V/V.

[2]The sample of fast green FCF used was a product of the National Aniline and Chemical Company, Inc., with a 97% total dye content. It was certified, No. NGf–7 by the Commission on Biological Stains. The closely related light green SF Y, C.I. No. 670, was tried and found effective as a mitochondrial colorant.

4. They are counterstained with a 1% (W/V) solution of safranin[3] in 50% ethanol for 3 to 6 minutes.

5. The slides are passed through graded ethyl alcohols into an equal mixture of ethyl alcohol and xylene and finally into xylene before mounting in clarite.

The duration of exposure to the action of 70% and 95% ethyl alcohol has no appreciable effect on the tenacity of the fast green to the mitochondria. It has, however, a determining influence on the quantity of safranin removed and is preferably controlled with the microscope so that the elution of safranin is adequate but not excessive. The immersion of the slides in picric acid was found essential to the procedure, as otherwise the safranin was too tenaciously bound for satisfactory removal by the alcohols. The steadfastness of the fast green FCF is unimpaired even after action by the ethanol for 45 minutes.

The technic was applied to different tissue homogenates prepared by several procedures (Green, Loomis and Auerbach, 1948, and Schneider and Potter, 1949) and derived from rabbit kidney, liver, pancreas and heart muscles. In each, the mitochondria were uniformly spherical and contained a discernible complexity of structure which had been regularly observed by phase contrast. The stain clearly demarcated the crescentric dense zone and the elliptical residual area. Such differentiation was not obtained by Altmann's (1894) acid aniline fuchsin technic, by which the units are imparted a dense solid appearance. On the other hand, tissues obtained from several species (mouse, rat and rabbit) were stained by the present method, after fixation by either Altmann's (1894) or by Regaud's (1909) method. In all the tissues studied, i.e., heart, liver, kidney and pancreas, the mitochondria, which maintained their rod shape, acquired the green stain selectively; the only additional structures which absorbed the dye in perceptible concentration were the erythrocytes, the nucleoli and plasmosomes. All these stained a brilliant grass green, as compared with the mitochondria which were a darker bluish-green hue. The mitochondria were sharp and distinct; in the pancreas, for example, the separation of them from the secretory granules was readily effected. In the kidney sections of rats and mice, which had been injected with either uranium salts or with alloxan, the cells of the proximal convoluted tubules were selectively damaged and contained swollen, spherical mitochondria of altered tinctorial intensity. In all sections the safranin served as a suitably contrasting nuclear and cytoplasmic stain.

[3]Two varieties of safranin were used. One sample had been obtained from Grübler, Leipzig, and was of unknown total dye content. It proved very satisfactory. The other was a National Aniline and Chemical Company product called Safranin–O, C.I. No. 841, with a 94% dye content. This differed slightly from the Grübler dye in its resistance to differentiation and required a longer period of washing by alcohol to elute it satisfactorily.

It is believed that this staining technic has opened avenues of considerable theoretical significance and much practical value. The selection of the particular dye depended entirely upon the peculiar practical rules derived from a critical analysis of extant classical methods for staining mitochondria. The immediately successful application confirmed the enunciation of those requirements. This indicates that the affinity of the dyes for mitochondria and erythrocytes is dependent inter alia on the chemical configuration of the dye. The adjuvant requirements of heat and the use of aniline implement the mechanism as a chemical rather than a physical process. It is conceivable that the aniline and the heat facilitate a close bond between specifically reactant groups in both the dye and the mitochondria much in the manner of Ehrlich's side chain reaction. On the other hand, the chief practical value of this technic depends upon its rapidity, certainty and simplicity. The technic is easily manipulated and requires no fastidious or exacting attendance upon problems of overstaining and differentiation. Such capriciousness and fugacity as have characterized the technics of Benda (1901) and Altmann (1894) have not been encountered in the present studies. Differentiation is the chief weak point of both procedures, as Benda himself has suggested (Benda, 1901). It is important that the differentiation with the present technic affects only the counterstain and exerts no solvent action upon the mitochondrial stain, whereas in previous methods the process of differentiation ultimately affected the intensity of the mitochondria. This substantiates the greater selectivity and specificity of aniline fast green FCF as a mitochondrial stain. Specimens stained by this procedure have (to date) remained unfaded for a period of three months.

REFERENCES

ALTMANN, R. 1894. Die Elementorganismen und ihre Beziehungen zu den Zellen. Veit et Co., Leipzig. See p. 33.

BENDA, C. 1901. Die Mitochondriafärbung und andere Methoden zur Untersuchung der Zellsubstanzen. Anat. Anz. Ergänzhft., **19**, 155–74.

BENSLEY, R. R. 1911–1912. Studies on the pancreas of the guinea pig. Amer. J. Anat., **12**, 297–388.

GREEN, D. E., LOOMIS, W. F., and AUERBACH, V. H. 1948. Studies on the cyclophorase system. I. The complete oxidation of pyruvic acid to carbon dioxide and water. J. Biol. Chem., **172**, 389–403.

KULL, H. 1913. Eine Modifikation der Altmann'schen Methode zum Färben der Chondriosomen. Anat. Anz., **45**, 153–7.

PETERSON, A. R., CONN, H. J., and MELIN, C. G. 1934. Methods for the standardization of biological stains. The triphenylmethane derivatives. Stain Techn., **9**, 41–8.

REGAUD, C. 1909–1910. Études sur la structure des tubes seminifères et sur la spermatogenese chez les mammifères. Arch. d'Anat. Micr., **11**, 291–431.

SCHNEIDER, W. C., and POTTER, V. R. 1949. Intracellular distribution of enzymes. IV. The distribution of oxalacetic oxidase activity in rat liver and kidney fractions. J. Biol. Chem., **177**, 893–903.

VOLKANSKY, M. 1928. Sur une nouvelle modification de le technique d'Altmann. Bull. d'Histol. Appl., **5**, 220–2.

THE INCREASED BASOPHILIA OF TISSUE PROTEINS AFTER OXIDATION WITH PERIODIC ACID[1]

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ABSTRACT.—**Oxidation by periodic acid (a 1% aqueous solution for 1 hour at 37° C.) greatly enhances the basophilic staining normally present in the keratinized portions of epidermis and hair. It also induces strong basophilia in purified fibrin and in the cytoplasm of smooth and striated muscle fibers, endothelium and the cells of the pancreatic islets and acini. Proof is offered that this observed basophilia differs from that of either acid mucopolysaccharides or nucleoproteins. The conclusion is reached that, besides revealing polysaccharides occurring in tissue sections, periodic acid also brings about the formation of strongly acid, basophilic groups. These acid groups have not been identified, but since they may be formed most readily in regions of high sulfur content, it is suggested that sulfide and sulfhydryl groups may be oxidized to the corresponding sulfonic acids which could account for the observed characteristics of the basophilic substances.**

Periodic acid has been introduced recently into histology as a reagent for oxidizing carbohydrate residues in compounds such as glycogen and the mucoid substances. The rationale for its use is that periodic acid breaks a carbon chain between atoms bearing adjacent hydroxyl or hydroxyl and amino groups, with the formation of a terminal aldehyde which can be identified by the Schiff leucofuchsin reagent. The procedure, now generally known as the periodic-acid-Schiff method, was developed by McManus (1946), Lillie (1947) and Hotchkiss (1948). These authors as well as Gersh (1947, 1948a, 1948b), Catchpole (1947, 1949) and Wislocki and Dempsey (1948) have found the procedure a valuable one for demonstrating glycogen, collagenous reticulum, fibrin, elastic fibers, various mucoid substances and certain intracellular glucoproteins. The latter include proteins from the anterior pituitary gland having the solubilities of gonodotrophic hormones (Catchpole, 1947) and from the kidney having the solubilities of renin (Marshall and Wakerlin, 1949).

The oxidation of polysaccharide substances is an interesting and valuable reaction, but other effects can also be produced in tissues

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by periodic acid. It also increases greatly the affinity of pure proteins and of tissue components for basic dyes. The following account is a description of the altered reactions with acid and basic dyes which have been encountered in both tissue proteins and a purified protein treated with periodic acid prior to staining.

Material and Methods

Various tissues and fibrin film, a solid, purified protein, were used. Skin and hair were obtained from the abdominal region of

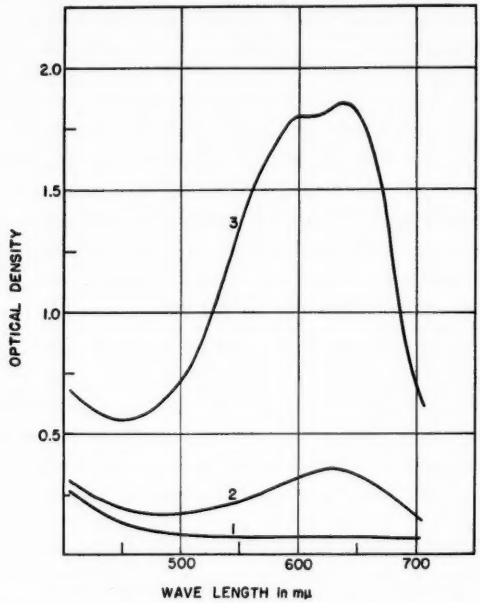

Fig. 1.—Absorption spectra of pieces of fibrin film mounted in clarite on glass slides. Curve 1 represents film fixed in Zenker's fluid and oxidized in periodic acid, but unstained. Curve 2 is from a piece of fixed film, unoxidized and stained with toluidine blue at pH 4.12. Curve 3 is from fixed and oxidized film, stained at pH 4.12. Oxidation increases greatly the amount of dye bound by the film. The greenish color of the stained film is attributable to the yellowish color of the unstained protein, mixed with the blue color of the dye. Despite the high concentration of dye represented in curve 3, the absorption peak at 560 mμ characteristic of metachromasia is absent.

albino rats and from the velvet covering of the growing antlers of the Virginia deer. Pancreas and liver were secured from a young Rhesus monkey. Blocks of tissue, comprising the trachea, esophagus, thyroid gland, striated muscle and the associated blood vessels, nerves and connective tissues were obtained from the neck region of rats. Unmodified fibrin in the form of a thin film was prepared from fibrinogen extracted from human blood by the Plasma Frac-

tionation Laboratory of Harvard University. This protein, prepared as a purified fibrin film and treated as a histological section, served the purpose of a model tissue.

The tissues were fixed in Zenker's fluid for 24 hours and then washed, dehydrated, embedded in paraffin and sectioned at 5μ. Deparaffinized sections mounted on glass slides were immersed in a 1% aqueous solution of periodic acid ($HIO_4 \cdot 2H_2O$ Eimer & Amend) for one hour at 37° C. They were then washed in several changes of water and stained by one of the following methods: with eosin-methylene-blue (Mallory, 1938); in a 0–5% aqueous solution of toluidine blue, a basic dye; in a 1% aqueous solution of light green, an acid dye, or with methylene blue, another basic dye, under carefully regulated conditions of pH according to the procedures of Dempsey and Singer (1946) and Singer and Morrison (1948). A control section, not previously treated with periodic acid, was also stained simultaneously with all of the above procedures. After staining, the sections were dehydrated, cleared and mounted in clarite.

Pieces of fibrin film (approximately 1 cm. square) were fixed for one hour in Zenker's fluid, and then washed overnight. Without embedding, they were then stained with toluidine blue in a 0.5% aqueous solution and with methylene blue following the method using controlled pH. The preparations were then dehydrated, cleared and mounted in clarite on glass slides.

The degree and characteristics of staining were evaluated visually with the microscope and measured and recorded as graphs with the microphotometer described by Dempsey, Bunting, Singer, and Wislocki (1947) as well as from absorption spectra with a Beckman spectrophotometer.

In addition to periodic acid, other oxidizing agents were employed. Sections were immersed in a 0.25% aqueous solution of potassium permanganate or a 4% aqueous solution of chromic acid for one hour at 37° C.

Results

After oxidization with periodic acid, the affinity of fibrin for the basic dye toluidine blue was greatly increased as is shown in Fig. 1 in which the absorption spectra of stained films are shown before (2) and after oxidation (3).

Toluidine blue belongs to a class of basic dyes which exhibits the phenomenon of metachromasia. In concentrated solutions and when bound to certain substances, the dye molecules polymerize and the color shifts from the euchromatic blue to a metachromatic purple. When stained with toluidine blue, fibrin film assumed a cold greenish blue color. Except for the absorption below 450 mu-

the spectrum of the stained film was characteristic of that of the euchromatic color of the dye (Michaelis, 1947). These results are in harmony with those of Ferry, Singer, et al (1947) and Singer and Morrison (1948) who found that Beer's law was obeyed over a considerable range of concentration when a similar dye, methylene blue, was used as a stain for fibrin film. The absorption below 450 mμ, which accounted for the greenish cast of the stained film, was caused not by a change in the absorption characteristics of the bound dye but rather by the yellowish color assumed by the unstained film after treatment with Zenker's fluid (Fig. 1).

Tissue sections also exhibited greatly increased basophilia after oxidation in periodic acid, and like the film, the tissue components bound the dye in its euchromatic blue form. Some components were influenced more than others. The hair shaft, which normally does not attract basic dyes, stained intensely after oxidation. The islets of Langerhans and the cells of pancreatic acini became exceedingly basophilic. Collagenous fibers and the endothelium of cutaneous blood vessels were other elements noteworthy for the increase in their dye-binding capacities. Figs. 2 and 3 illustrate comparable fields of skin and hair before and after oxidation, and Figs. 6 and 7 show the effect of treatment with periodic acid upon the pancreas. In striated muscle, a patchy irregular staining with basic dyes is the rule. The majority of the fibers ordinarily are eosinophilic, but especially along the cut edges of the block the myofibrils often are diffusely stained and exhibit strong basophilia in the anisotropic bands and in the Z discs. After oxidation with periodic acid, the basophilia of muscle was no longer patchy. The Z discs and the anisotropic segments were stained selectively and intensely no matter where they were located in the section.

Unlike its effect on basophilia, periodic acid decreased the affinity of most tissues for the acid dye, light green. This decreased acidophilia was especially noteworthy in collagen fibers which normally stain brilliantly with light green but after periodic acid were almost totally unstained. On the other hand, the smooth muscle cells of blood vessels and in the pancreatic ducts were unaffected and continued to stain strongly after oxidation.

FIG. 2.—Skin and hair from the velvet covering the growing antler of a Virginia deer. The tissue was fixed in Zenker's fluid and the section stained with methylene blue at pH 5.7. ×90.

FIG. 3.—Section adjacent to that shown in Fig. 2. The section was oxidized in periodic acid prior to staining with methylene blue at pH 5.7. The epidermis, derma and hair exhibit greatly increased basophilia.

FIG. 4.—Section adjacent to that shown in Fig. 2. This section was subjected to hydrolysis in hydrochloric acid, then oxidized in periodic acid, and finally stained with methylene blue at pH 5.7. Acid hydrolysis does not destroy the substance oxidized by the periodic acid. ×90.

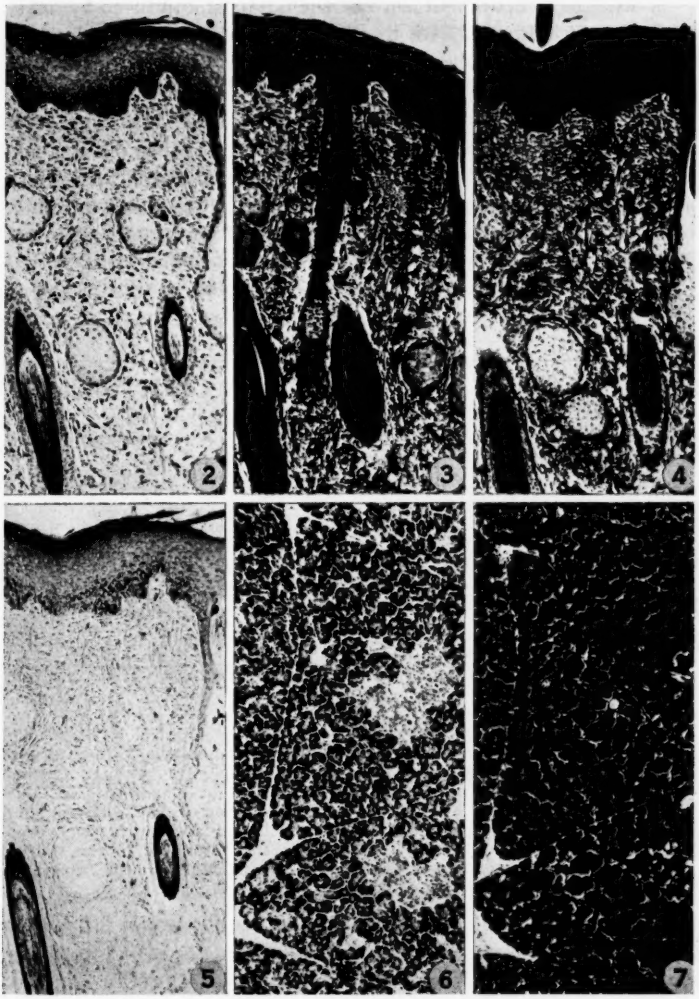

FIG. 5.—Section adjacent to that shown in Fig. 2. This section was subjected to acid hydrolysis and then stained with methylene blue at pH 5.7. The residual dark material in the stratum germinativum is the melanin pigment of the skin. Acid treatment destroyed the basophilic substances of the stratum germinativum and of the nuclei, but did not affect that in the cornifying layers of skin and hair. ×90.

FIG. 6.—Section of pancreas from a Rhesus monkey. The tissue was fixed in Zenker's fluid and the section stained with methylene blue at pH 5.7. ×90.

FIG. 7.—Section adjacent to that shown in Fig. 6. This section was oxidized in periodic acid prior to staining with methylene blue at pH 5.7. The islets of Langerhans and the acinar cells show greatly increased basophilia. ×90.

In order to compare and contrast the basophilia induced by oxidation with periodic acid with that known for various other elements, a further set of experiments was initiated. Sulfuric and phosphoric groups are responsible for the basophilia of acid mucopolysaccharides and nucleoproteins, respectively. These groups exist as ester linkages which may be broken and the basophilia destroyed by hydrolysis in mineral acids. Treatment of sections in hydrochloric acid (1*N* for 3 hours at 37° C.) destroyed the basophilia attributable to nuclear and cytoplasmic nucleoproteins. Fig. 5, when compared with Fig. 2, illustrates the destructive effect of hydrochloric acid upon this basophilia. The sulfate-containing mucopolysaccharides can also be destroyed by acid hydrolysis, but in the case of cartilage

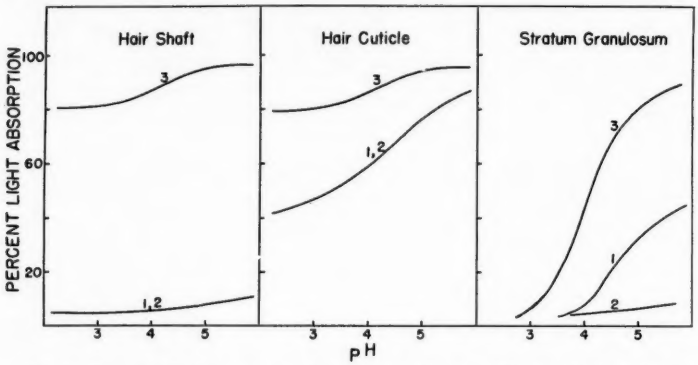

FIG. 8.—Curves relating uptake of methylene blue to pH after hydrolysis and oxidation of hair and skin. Curve 1 describes the dye uptake in sections fixed in Zenker's fluid and subjected to no other treatment prior to staining. Curve 2 describes staining in similar preparations subjected to acid hydrolysis and then stained. Curve 3 represents the effect of oxidation in periodic acid prior to staining. Acid treatment is without effect upon the hair shaft and cuticle (the cornifying zone illustrated in Fig. 2–5), but destroys the basophilia of the stratum granulosum which is attributable to nucleoprotein (Cf. Fig. 2 and 5). Oxidation greatly enhances the basophilia of all three regions (Cf. Fig. 2 and 3).

and mucus, somewhat longer treatments are necessary. Such hydrolysis did not abolish the strong basophilia induced by oxidation in periodic acid nor that normally occurring in cornifying regions of the hair shaft and epidermis (Fig. 4).

The effects of acid hydrolysis and of oxidation with periodic acid were determined with the microphotometer upon sections of hair and skin. In Fig. 8, the degree of staining (percent light absorption) is plotted against the pH at which staining was carried out according to the procedures described by Dempsey, Bunting, Singer, and Wislocki (1947). Curve 1 represents the affinity of control sections for the basic dye methylene blue, Curve 2 that of sections subjected to acid hydrolysis and Curve 3 that of sections oxidized by periodic

acid. In the hair shaft and cuticle, hydrolysis was without effect upon the staining properties (Fig. 5), but oxidation increased the dye-uptake markedly (Fig. 3). In the stratum granulosum, hydrolysis destroyed the initial basophilia presumably attributable to nucleoproteins, whereas oxidation was followed by more intense staining. The residual dark color in the stratum germinativum illustrated in Fig. 5 is caused by the granules of melanin pigment of the skin and should not be confused with basophilic staining which is completely suppressed.

Oxidizing agents other than periodic acid, such as potassium permanganate and chromic acid, increased the basophilia of tissue elements in a similar manner.

Discussion

Mucopolysaccharides and nucleoproteins are the two principal chemical classes of substances in tissues which are known to exhibit strong basophilia. The newly described basophilia induced by oxidation with periodic acid and that normally present in the cornifying portions of epidermis and hair cannot be attributed to either of these compounds. The acid mucopolysaccharides stain metachromatically and therefore differ from the newly described basophilic components which stain euchromatically. Moreover, the basophilia of both mucopolysaccharides and nucleoproteins can be destroyed by acid hydrolysis whereas the new type of basophilia is unaffected. These facts indicate that acid groups, differing from the sulfuric and phosphoric ester linkages, exist normally in cornifying epithelia and can be produced by oxidation of other structures.

The present data are not sufficient for identifying the acid groups responsible for the newly described basophilia. However, a slight clue is provided by the fact that the most intense basophilia of this character occurs normally as well as upon oxidation, in the keratinizing parts of skin and hair and to lesser extent in the islets of Langerhans, the endothelium of blood vessels and striated muscle. Of these various tissues, skin and hair have a high sulfur content, while the pancreatic islets secrete insulin, which is a sulfur-containing compound. Moreover, the endothelium of small blood vessels contains sulfhydryl groups (Bennett, 1948), while muscle possesses extractable substances similar to glutathion. Within the limits of these sketchy data, one can therefore conclude tentatively that the newly described basophilia is related to the sulfur content of proteins. Oxidation of sulfide and sulfhydryl groups leads to the formation of sulfonic acids (Suter, 1944). This last observation is particularly interesting inasmuch as the data, obtained by staining

at low pH values, indicate that the unidentified acid groups dissociate as strongly as do some mineral acids. Sulfonic acids are relatively strong acids (Conant, 1939) and therefore could account satisfactorily for the position of the pH signatures.

REFERENCES

BENNETT, H. S. 1948. The disposition of sulfhydryl in resting striated muscle. Anat. Rec., **100,** 100 (Supplement).

CATCHPOLE, H. H. 1947. Cellular distribution of glycoprotein in the anterior lobe of the pituitary gland. Fed. Proc., **6,** 88.

———— 1949. The ground substance of tumors and intravital staining. Anat. Rec., **103,** 17 (Supplement).

CONANT, J. B. 1939. The Chemistry of Organic Compounds. Revised Edition. Macmillan Co., New York.

DEMPSEY, E. W. and SINGER, M. 1946. Observations on the chemical cytology of the thyroid gland at different functional stages. Endocrinology, **38,** 270.

DEMPSEY, E. W., SINGER, M., BUNTING, H., and WISLOCKI, G. B. 1947. The dye-binding capacity and other chemo-histological properties of mammalian mucopolysaccharides. Anat. Rec., **98,** 417.

FERRY, J. D., SINGER, M., MORRISON, P. R., PORSCHE, J. D., and KUTZ, R. L. 1947. Preparation and properties of serum and plasma proteins. X. Modification of the physical and chemical properties of fibrin film by heat treatment. J. Amer. Chem. Soc., **69,** 409.

GERSH, I. 1947. Polysaccharide complex in individual follicles in the thyroid gland of the rat. Fed. Proc., **6,** 392.

———— 1948a. Histochemical studies of basement membrances. Fed. Proc., **7,** 270.

———— 1948b. Glycoprotein associated with the Golgi apparatus of certain cells. Anat. Rec., **100,** 32 (Supplement).

HOTCHKISS, R. D. 1948. A microchemical reaction resulting in the staining of polysaccharide structures in fixed tissue preparations. Arch. Biochem., **16,** 131.

LILLIE, R. D. 1947. Studies on the preservation and histologic demonstration of glycogen. Bull. Internatl. Assn. Med. Museums, **27,** 23.

LILLIE, R. D. 1948. Histopathologic Technic. Blakiston, Philadelphia.

MCMANUS, J. F. A. 1946. Histological demonstration of mucin after periodic acid. Nature, **158,** 202.

MALLORY, F. B. 1938. Pathological Technique. Saunders Co., Philadelphia.

MARSHALL, J., and WAKERLIN, G. E. 1949. Purification and possible histochemical localization of renin. Fed. Proc., **8,** 106.

MICHAELIS, L. 1947. The nature of the interaction of nucleic acids and nuclei with basic dyestuffs. Cold Spring Harbor Symp. on Quant. Biol., **12,** 131.

SINGER, M., and MORRISON, P. R. 1948. The influence of pH, dye and salt concentration on the dye binding of modified and unmodified fibrin. J. Biol. Chem., **175,** 133.

SUTER, C. M. 1944. The Organic Chemistry of Sulfur. Tetracovalent sulfur compounds. John Wiley and Sons, New York.

WISLOCKI, G. B., and DEMPSEY, E. W. 1948. The chemical histology of human placenta and decidua with reference to mucoproteins, glycogen, lipids and acid phosphatase. Amer. J. Anat., **83,** 1.

AN IMPROVED HISTOCHEMICAL TECHNIC FOR ACID PHOSPHATASE[1]

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ABSTRACT.—**Causes of failure in the histochemical technic for acid phosphatase were studied. It was found that the ratio of buffer to substrate in the incubating mixture is an important factor in the constancy of results. In the case of glycerophosphate as a substrate, the optimal ratio is 5 to 6; with other substrates it may be different. The mechanism of the effect of this ratio on the results is explained.**

The capriciousness of the technic for acid phosphatase (Gomori, 1941) has been reported repeatedly (Moog, 1943 and 1944; Hard and Lassek, 1946; Tissières, 1948, and others). There can be no doubt that considerable inactivation occurs during fixation and embedding (Stafford and Atkinson, 1948; Doyle, 1948; Cappelin, 1947; Rabinovitch, Junqueira and Fajer, 1949). This inactivation may affect the tissue in a more or less uniform or patchy way; sometimes, a layer at a certain distance from the surface shows the best preservation of enzymatic activity (Wolf, Kabat and Newman, 1943). However, there are phenomena which cannot be explained by inactivation in the course of fixation and embedding, or by variations in the composition of the glycerophosphate used (Sulkin and Kuntz, 1947). In this category belong the curious failures one may get with some but not all sections cut serially from the same paraffin block, or failures obtained with a fresh batch of incubating fluid, prepared from exactly the same ingredients as a previous successful one.

In an attempt to improve the reliability of the method, the effects of a number of factors, likely to have an influence on the results during incubation proper, were systematically examined. Four different paraffin blocks, each containing a number of acetone-fixed tissues, were used; consecutive serial sections were incubated with mixtures of varying composition, prepared as accurately as possible, because it was suspected that minor variations may cause an "all or none" effect. The pH of all experiments was kept at 5.0. In the first

[1]This work has been done under grants from the Douglas Smith Foundation for Medical Research of The University of Chicago, and from the Pathology Study Section of the U. S. Public Health Service.

series of experiments, Eastman's sodium glycerophosphate·$5H_2O$, catalog No. 644, was used.

Effect of substrate and buffer concentration. All ingredients of the original formula were kept constant, except for the amount of substrate. The stock solution was diluted with 2 parts of distilled water. With amounts of substrate much higher than given in the formula, copious precipitates of lead glycerophosphate were obtained; they were permitted to settle out (this process can be accelerated by gentle heating), and the clear filtrate was used. Under these conditions (buffer, 0.08*M;* lead, 0.0037*M* in the final solution), uniformly good results were obtained with substrate concentrations of 0.014*M* to 0.05*M;* with a substrate concentration of 0.005*M,* as called for in the original formula, results were erratic in that in some cases the results were excellent, in others, patchy or negative; below 0.003 they were almost invariably negative. The experiment was repeated by varying the concentration of the substrate at different

TABLE 1. TTHE SOLUBILITY OF LEAD PHOSPHATE IN ACETATE BUFFERS pH5 OF VARIOUS CONCENTRATIONS AND IN THE PRESENCE OF GLYCEROPHOSPHATE

Suspending medium	μg of P in 10 ml.
Dist. water	19
M/40 buffer	40
M/20 buffer	46
M/10 buffer	52
M/5 buffer	62
M/10 buffer + M/100 glycerophosphate	41
M/10 buffer + M/50 glycerophosphate	23

levels of buffer concentration. It was found that for uniformly good results, the concentration of the buffer must not exceed that of the substrate by a factor of more than 6; going below a ratio of 5 does not further improve results.

Effect of concentration of lead. Increasing the concentration of lead, with substrate and buffer concentration unchanged, also tends to increase the intensity and uniformity of the reaction but to a lesser extent and within narrower limits than increasing the concentration of substrate. With high concentrations of lead (lead/substrate greater than ½), the reaction actually becomes feebler.

The mechanism of these effects is probably as follows: Poorly soluble salts are usually more soluble at higher ionic strength. This applies to lead phosphate, too. The dependence of its solubility on the concentration of the buffer was determined by suspending an excess of carefully washed lead phosphate in buffers of various concentrations; the suspensions were allowed to stand at room tempera-

ture for 1 hour; they were shaken every 10 minutes. After 1 hour they were filtered, and phosphate was determined in 10-ml. samples. Table 1 shows the results obtained.

It is obvious that the solubility of lead phosphate increases with increasing buffer concentration; the depression of solubility by the presence of glycerophosphate is also clearly shown. On the basis of the mass law, increase in the concentration of lead will have a similar depressing effect on solubility.

In a previous paper (Gomori, 1949) the importance of the ratio of phosphate production to that of diffusion has been emphasized. It is clear that for optimal results this ratio should be as high as possible. The rate of hydrolysis in the case of acid phosphatase increases up to a substrate concentration of 0.1*M* (Gutman and Gutman, 1938). Therefore, it is advisable to keep substrate concentration high and, if possible, to use substrates which are hydrolyzed at a fast rate; solubility of the reaction product should be kept down as far as possible; for this reason, one should use the minimum amount of buffer necessary for adequate buffering. Raising the concentration of lead will depress the concentration of glycerophosphate ions; therefore, a low concentration of lead must be used. In the case of glycerophosphate as a substrate, the composition of an optimal incubating mixture is: buffer, 0.05*M;* substrate, 0.01*M,* and lead, 0.004*M*. It is suggested that two stock solutions be kept handy: I, 0.05*M* acetate buffer pH 5, containing 1.2 g. of $Pb(NO_3)_2$ per liter; II, a 0.1*M* (about 3%) solution of sodium glycerophosphate. Both stock solutions are stable in the ice box, with a few crystals of thymol or camphor added. For use, one adds 5 ml. of stock solution II to 50 ml. of stock solution I, and clears mixture as described.

New substrates. Another way was attempted to increase the efficiency of the method, the use of substrates hydrolyzed faster than glycerophosphate. It is known that phenylphosphate is hydrolyzed faster by acid phosphatase of the serum than glycerophosphate (Behrendt, 1943 and 1949); in fact, the enzyme of red cells attacks glycerophosphate poorly while it hydrolyzes phenylphosphate energetically. Unfortunately, the lead salt of phenylphosphate is so insoluble that its use in histochemistry is out of question. However, when the monophosphates of catechol, resorcinol and hydroquinone (Gomori, 1949) were prepared, it was found that they are hydrolyzed at a rate comparable to that of phenylphosphate (Table 2); in addition, their lead salts are sufficiently soluble for use in histochemistry.

Good results were obtained with the phosphates of catechol and

resorcinol; hydroquinone was found unsuitable as a histochemical substrate. The optimal buffer/substrate ratios are: catecholphosphate, 35; resorcinolphosphate, 12. These high ratios are explainable by the rapid rate of hydrolysis of these substrates. The pictures obtained with their use are essentially the same as seen with the original method, except that with resorcinolphosphate the contrasts are less sharp; *e.g.* glomeruli of the kidney do not stain as dark as they do with the other substrates. It was expected that with the use of the aromatic substrates an intense staining of the red cells would be obtained; however, this was not observed.

On the basis of these results a few more substrates which gave poor results on previous attempts were tried once more. Good results were obtained with the following, at the specified buffer/substrate ratios: adenosinetriphosphate, 15; glucose–6–phosphate, 6, and fructose–6–phosphate, 6. The pattern of distribution of enzy-

TABLE 2. RATES OF HYDROLYSIS OF VARIOUS PHOSPHATE ESTERS BY ACID PHOSPHATASE

(Expressed in micromoles of substrate hydrolyzed per hour; glycerophosphate = 100)

Source of enzyme	Phosphate ester of				
	Glycerol	Phenol	Catechol	Resorcinol	Hydroquinone
Human serum.....	100	310	230	280	330
Human prostate....	100	170	100	125	110
Spleen (guinea pig)	100	280	290	280	220
Liver (guinea pig)..	100	350	300	340	330

matic activity as seen with the sugar phosphates is the same as with glycerophosphate; with adenosinetriphosphate, red cells and muscle also react quite intensely and, in addition, there are also other minor differences seen.

Since the new substrates and the sugar phosphates offer no advantage over the easily available and cheap glycerophosphate, there seems to be no indication for their use; adenosinetriphosphate, on the other hand, deserves further study.

REFERENCES

BEHRENDT, H. 1943. Phosphatase activity of human erythrocytes. Proc. Soc. Exp. Biol. and Med., **54**, 268–71.

BEHRENDT, H. 1949. Phosphatases in blood of man. Amer. J. Clin. Path., **19**, 167–76.

CAPPELIN, M. 1947. Une technique pour le dosage cytochimique des phosphatases. Bull. d'hist. appl., **24**, 155–62.

DOYLE, WM. L. 1948. Effects of dehydrating agents on phosphatases in the lymphatic nodules of the rabbit appendix. Proc. Soc. Exp. Biol. and Med., **69,** 43–4.

GOMORI, G. 1941. Distribution of acid phosphatase in the tissues under normal and under pathologic conditions. Arch. Path., **32,** 189–99.

GOMORI, G. 1949. Histochemical specificity of phosphatases. Proc. Soc. Exp. Biol. and Med., **70,** 7–11.

GOMORI, G. 1949. Further studies on the histochemical specificity of phosphatases. Proc. Soc. Exp. Biol. and Med. *In press.*

GUTMAN, A. B. and GUTMAN, E. B. 1938. An acid phosphatase occuring in the serum of patients with metastasizing carcinoma of the prostate gland. J. Clin. Invest., **17,** 473–8.

HARD, W. L., and LASSEK, A. M. 1946. The pyramidal tract. Effect of maximal injury on acid phosphatase content in neurons of cats. J. Neurophysiol., **9,** 121–6.

MOOG, F. 1943. The distribution of phosphatase in the spinal cord of chick embryos of one to eight days incubation. Proc. Natl. Acad. Sci., **29,** 176–83.

RABINOVITCH, M., JUNQUEIRA, L. C., and FAJER, A. 1949. A chemical and histochemical study of the technic for acid phosphatase. Stain Techn., **24,** 147–56.

STAFFORD, R. O., and ATKINSON, WM. B. 1948. Effect of acetone and alcohol fixation and paraffin embedding on activity of acid and alkaline phosphatases in rat tissues. Science, **107,** 279–81.

SULKIN, N. M., and KUNTZ, A. 1947. The sodium glycerophosphate factor in the technic for phosphatase activity. Anat. Rec., **99,** 639–40.

TISSIÈRES, A. 1948. L'activité des phosphomonoestérases et des pyrophosphatases dans le rein et l'intestin du rat surrénal-ectomisè et l'action du dèsoxycorticostérone. Acta Anat., **5,** 224–34.

WOLF, A., KABAT, E. A., and NEWMAN, WM. 1943. Histochemical studies on tissue enzymes. III. A study of the distribution of acid phosphatases with special reference to the nervous system. Amer. J. Path., **19,** 423–39.

PREPARATION OF COPOLYMERS OF ISOBUTYL METHACRYLATE AND STYRENE FOR MOUNTING MEDIA

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ABSTRACT.—**Details are given for the preparation of low molecular weight copolymers of isobutyl methacrylate and styrene for use in mounting microscopical sections between the slide and cover glass. Any refractive index in the range from 1.477 to 1.590 is available, depending upon the proportions of monomers used. A refractive index of 1.550 is recommended for general purposes, though other indices might be desirable in special cases including phase microscopy. The resin gives solutions in toluene of suitable fluidity at usable concentrations, adheres satisfactorily to glass, and has excellent light-transmission. The features which make it especially valuable are its optimum refractive index and its permanent water-whiteness.**

Copolymers of isobutyl methacrylate and styrene may also be used to prepare plastic mounts of thick sections or gross specimens, which are optically cleared by the plastic mountant.

Eleven years ago, at the time of introducing the Clarites (Nevillites) as media for mounting sections between the slide and cover glass (Groat, 1939), I was engaged in an attempt to adapt certain of the vinyl type of polymers, then new, to the same purpose. The major objection to some of these polymeric resins which seemed otherwise satisfactory, lay in the difference between their refractive index and the refractive index of most of the stained tissue elements commonly examined microscopically (Groat, 1940). Another interfering factor was the great viscosity of even dilute solutions of the substances, referable to their exceedingly high molecular weight.

A low molecular weight copolymer of isobutyl methacrylate and styrene was prepared in an effort to meet the optical and solubility requirements of a useful mounting medium. Cover glasses were applied to a number of slides with this medium. One set of slides of the same tissue was prepared expressly for the purpose of comparing as mounting media, gum damar (Singapore damar No. 1), Clarite X (Nevillite No. 1), Clarite (Nevillite V), and the copolymer. This was in January, 1939.

The copolymer had a major shortcoming. It partially but rapidly faded hematoxylin stain. No effort was made to eliminate the effect on hematoxylin till about a year ago. In the interim, the copolymer was proving its superiority with respect to aging. At the present time, the comparison mount prepared with it is still perfectly water-white. The other three comparison mounts have yellowed very slightly, Clarite the most faintly, then Clarite X, and then gum damar.

The superlative aging characteristics of the copolymer medium prompted an attempt to prevent the fading of hematoxylin by it, and thereby make it suitable for general use. It was found that this can be accomplished either by employing a recently proposed hematoxylin stain (Groat, 1949) that is not affected by the unpurified copolymer, or by refining the copolymer preparation.

TABLE 1. SOME PHYSICAL PROPERTIES OF ISOBUTYL METHACRYLATE AND OF STYRENE

	Isobutyl methacrylate*		Styrene†	
	Monomer	Polymer	Monomer	Polymer
Boiling point	155°C.	——	145°C.	——
Density, g/ml at 25°C.	0.88	1.02	0.90	1.05
Refractive index	1.422	1.477	1.544	1.590

*From data published by the E. I. du Pont de Nemours & Company, Inc.
†From data published by The Dow Chemical Company.

TECHNICAL CONSIDERATIONS

The monomeric substances chosen for copolymerization were isobutyl methacrylate and styrene. Several considerations entered into this selection. They were based on the expectations, later borne out, that the properties of the copolymer would be an amalgamation of those of the polymers of the individual monomers employed. Isobutyl methacrylate polymer gives solutions of appreciably lower viscosity than comparable preparations of its congeners. It appears to adhere to glass slightly better than methyl methacrylate polymer, and has a higher thermal yield point than its related polyesters excepting methyl methacrylate. Both polymers are water-white, and have excellent light-transmission. The refractive indices are such that a copolymer with the proper refractive index for a mounting medium can be prepared. Certain salient data on the two compounds are given in Table 1.

It was found, empirically, that the refractive index of a copolymer of a methacrylate and styrene is, at least as a close approximation, the sum of the products obtained by multiplying the refractive index

of the pure polymeric phase of each constituent, by the volume-fraction it would occupy in the completed copolymer if this is considered, hypothetically, as an ideal solution of individual polymeric phases. In establishing this relation, refractive index was determined with a Smith refractometer, but subsequently, the refractive index was estimated by the immersion method (Groat, 1941).

By copolymerizing proper quantities of isobutyl methacrylate and styrene, a resin of any desired refractive index from 1.477 to 1.590 can be prepared. The refractive index selected for the mounting medium to be used for all ordinary purposes is 1.550, on the basis of considerations previously given (Groat, 1940). Other refractive indices in the range available might have some usefulness in phase microscopy, and wherever individual preference or need calls for them.

The molecular weight attained by a polymer of the vinyl type is influenced by certain conditions under which polymerization is effected. The molecular weight is decreased by increasing the polymerizing temperature, by increasing the concentration of catalyst, and by diluting the monomer (Strain, 1938). Application of these principles enabled production of a resin which gave a solution of sufficient concentration and fluidity to qualify it in these respects as a good mounting medium.

Preparation of Copolymer

The following directions are for preparation in the laboratory of small amounts of the copolymer mounting medium.

The volumes of the two monomers to be used to obtain a copolymer of any desired refractive index, n, between 1.477 and 1.590, can be determined from the following relationship:

$$\frac{\text{ml. isobutyl methacrylate monomer}}{\text{ml. styrene monomer}} = \frac{0.993\ (1.590-n)}{n-1.477}$$

This was derived on the basis of the statement made under 'Technical Considerations' concerning the refractive index of a copolymer, using the density data given in Table 1.

If, for example, it is desired to make approximately 100 g., dry weight, of copolymer having a refractive index of 1.550, one should place in a 500–ml., pyrex, round-bottom flask, 42 ml. of isobutyl methacrylate monomer[1], and 78 ml. of styrene monomer[2]. The

[1]From E. I. du Pont de Nemours & Company, Ammonia Department, Wilmington 98, Delaware.

[2]From Bakelite Corporation, 230 Grove St., Bloomfield, N. J.; or Dow Chemical Company, Midland, Michigan.

inhibitors which the manufacturers add to these monomers should not be removed. They are ineffective under the conditions of polymerization employed here. When not in use, the monomers should be stored in tightly closed containers in a cool, preferably refrigerated, place. This is important, for if polymer forms in the monomer during storage, a copolymer prepared using the monomer may not be homogeneous inasmuch as the individual polymers have a limited solubility in the copolymer.

A volume of xylene equal to the combined volumes of the monomers (in this example, 120 ml.) is then added to the flask; and 0.4 g. benzoyl peroxide per 100 ml. of monomers (in this case, 0.48 g.), is added to catalyze the copolymerization. The contents of the flask are swirled to dissolve the catalyst. The flask is stoppered with a cork covered with aluminum foil, and placed in an oil bath (e.g., cottonseed oil) preheated on an electric hot plate to a constant temperature range of 137° C. to 142° C. (The boiling range of xylene falls within this temperature span.) The top surface of the contents of the flask should be about level with the top surface of the oil in the bath. The temperature of the bath should be registered continuously by a thermometer, the bulb of which is well beneath the surface of the oil and touching or nearly touching the flask. It will at first drop a few degrees, but if the heating arrangement is not disturbed, the temperature will reach the previous range in a few minutes. When the temperature begins to rise after the initial drop, the stopper should be removed momentarily from the flask to allow the heated air to escape, and then, replaced very tightly. The bath temperature should be maintained at the range of 137° C. to 142° C. throughout the process of copolymerization. Caution should be observed in working with these volatile, inflammable liquids. The wearing of plateglass glasses, or goggles is advisable when attending the heated flask.

The flask is allowed to remain in the bath for 48 hours. It may be removed sooner, but the yield of copolymer will be lower. The polymerization time is the same, regardless of the size of the batch being prepared. The flask is taken from the bath, and may either be cooled slowly at room temperature, or rapidly in cold water. The stopper should be loosened as cooling gets under way; otherwise, the pressure differential produced may make the cork very difficult to remove.

In the following processing it is not necessary to measure the volumes of fluids used.

The contents of the flask are poured into a large beaker. An

equal volume of acetone is added. This acetone may first be used to rinse out the flask. The solution is stirred until homogeneous. The copolymer is precipitated from the solution by adding to it, in the beaker, four times its volume of 95% ethyl alcohol. This should be poured in very slowly at first, with continual stirring, and then may be added more rapidly as the precipitate becomes heavy. Stirring collects the precipitate into a gummy mass. If some remains free as small, flocculent masses, or becomes attached to the wall of the beaker, it may be incorporated into the main bolus by pressing the latter against it with the stirring rod. As the mass is stirred and prodded with the stirring rod, it becomes firmer as solvent in it is replaced by 95% ethyl alcohol. The fluid is then decanted, and the mass should be prodded with the stirring rod to remove trapped fluid, which is then poured off.

When the trapped fluid has thus been removed, a sufficient volume of acetone is added to the beaker to redissolve the precipitate and give a solution of at least the same volume as that from which the copolymer was first precipitated. When solution is complete, the copolymer is reprecipitated with four volumes of 95% ethyl alcohol, the mass collected, prodded, the supernatant fluid poured off, and trapped fluid removed as before.

The precipitate is again dissolved in acetone, this time enough to give a dilute, low-viscosity solution. The solution should be dilute enough so that when it is poured very slowly into water, the copolymer precipitates immediately as a white, spongy, formation. It has been suggested that vinyl polymers be precipitated from acetone solution by pouring the solution into a fast stream of water in the laboratory trough (Werbin, 1948). However, it may be done very satisfactorily in the absence of a laboratory trough, and perhaps with less loss and chance of contamination. Water is placed in two containers, each holding a volume at least four times the volume of the solution. The solution is poured very slowly into the water in one of the containers, care being taken not to pour any solution over the precipitate, which rises to the surface. It is necessary to observe the precautions of having a dilute solution, of pouring slowly, and of pouring always into water, not onto floating resin, in order that the precipitate form properly as a white, very porous mass, not as a mass of solution walled off from the water by a coating of resin. When the surface of the water is covered with floating resin, a stirring rod is slanted beneath the layer, and the subsurface fluid is agitated to mix the acetone into the body of water. This done, the resin may be pushed to one side, but not pressed together, and more

solution poured into the water. When the surface becomes filled with resin, this is forced beneath the surface with the hand to remove any remaining concentration of solvent, and the precipitate is lifted into the second container of water. Precipitation is continued in the first container, the water being changed if necessary, and the resin being transferred to the second container. When all the resin is in the second container, the water is drained off and replaced by fresh water. This is drained off, and the resin is allowed to dry either at room temperature or in an oven below 60° C.

When copolymerization is carried out under the conditions outlined, 88% by weight of the monomers reacts by 24 hours, 97% by 48 hours. About 5% of the copolymer is lost subsequently, principally during the collection of the precipitate from water. The final yield, after a 48-hour polymerization period and subsequent purification, is thus about 92%.

The copolymer may be prepared by a somewhat different, and less satisfactory procedure. The monomers are mixed, the inhibitors removed by washing in a separatory funnel with several changes of dilute sodium hydroxide solution, the sodium hydroxide removed by washing well with water, and the monomers dried with anhydrous sodium sulfate. The process of copolymerization is the same as before, except that less catalyst is used. The solution of resin in xylene may be used as it is when the reaction is finished; but it fades hematoxylin stains, excepting iron hematoxylin (Groat, 1949).

Preparation and Use of Mounting Medium Solution

Solutions of the resin in toluene have as low a viscosity with respect to concentration as in any solvent; and toluene is preferred on account of its general inertness toward stains, and its favorable vapor pressure at the ordinary drying temperatures. Strain (1940) showed that the viscosity of methyl methacrylate polymer solutions in toluene is considerably reduced by replacement of a portion of the toluene by certain other liquids, notably methyl alcohol and 95% ethyl alcohol which are themselves non-solvents; but this does not hold for the copolymer of isobutyl methacrylate and styrene described here.

A solution of approximately 45% by weight of copolymer in toluene is recommended for use in covering sections. This may be prepared directly by dissolving 2 g. resin in 3 ml. toluene. For many purposes, however, it will be found desirable to prepare a somewhat more dilute solution, to run this through a retentive filter, and to allow the filtrate to concentrate sufficiently. The solution filters exceedingly slowly through a close paper such as Whatman No. 2.

and during this time the receptacle should be shielded against entry of dust by impaling a piece of paper on the stem of the funnel to guard the opening of the bottle or beaker, and the contents of the funnel should be protected against excessive evaporation by placing a petri dish over the top of it.

The resin prepared according to the preferred method is essentially pure. It has not faded hematoxylin or the numerous other stains it has been used on. Sections from 3μ to 50μ in thickness, taken out of xylene, have been routinely covered with it. Sections can be taken out of non-solvents for the copolymer, provided that the non-solvent will in time evaporate from under the cover slip, and will not extract the stain. For example, sections stained with hematoxylin and eosin can be dehydrated in isopropyl alcohol and covered directly from it. Low viscosity nitrocellulose need not be removed from sections prior to covering. The appearance of voids beneath the cover slip as the solvent evaporates indicates either that too little solution was applied, or that the solution was not sufficiently concentrated.

The copolymer has suitable solubility properties, adheres to glass satisfactorily, has optimum optical properties, and is permanently water-white on the slide. In addition, there is the valuable point that media of different refractive index can be prepared as required. It equals or surpasses the Clarites except in one characteristic: it softens at a lower temperature, and hence cannot be subjected to as high temperatures during microprojection.

Use as a Clearing Mountant for Gross Specimens

The beautiful and thoroughly useful museum mounts of biological specimens in transparent plastics are familiar objects. The refractive index range which is covered by copolymers of isobutyl methacrylate and styrene, makes it possible to prepare embedded objects and thick tissue sections which are optically cleared by the embedding plastic. Preliminary treatment of specimens and determination of the refractive index of the embedding medium which will give optimum clearing may be carried out as for optical clearing with fluids (Groat, 1941). Whatever method is used for the embedding, a tough copolymer should be prepared by mixing monomers, removing inhibitors, and copolymerizing at a relatively low temperature, no higher than 100° C. With small amounts of catalyst added, lower reaction temperatures should be used. In case of casting, finishing at higher temperatures is desirable to reduce the content of monomers, and to anneal.

REFERENCES

GROAT, R. A. 1939. Two new mounting media superior to Canada balsam and gum damar. Anat. Rec., **74,** 1–6.

GROAT, R. A. 1940. Evaluation of isobutyl methacrylate polymer as a mounting medium. Science, **92,** 268.

GROAT, R. A. 1941. Clearing tissue with mixtures of tributyl and tri-o-cresyl phosphates. Stain Techn., **16,** 111–17.

GROAT, R. A. 1949. Initial and persisting staining power of solutions of iron-hematoxylin lake. Stain Techn., **24,** 157–63.

STRAIN, D. E. 1938. Polymerization of methyl methacrylate in organic solvents. Ind. Eng. Chem. (Ind. Ed.), **30,** 345–7.

STRAIN, D. E. 1940. Viscosity variations in methacrylic ester polymer solutions. Ind. Eng. Chem. (Ind. Ed.), **32,** 540–1.

WERBIN, H. 1948. An improved method of precipitating and drying vinyl copolymers. Science, **107,** 400–1.

DIFFERENTIAL STAIN FOR THE HUMAN AND ANIMAL ANTERIOR HYPOPHYSIS

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ABSTRACT.—**A staining method is described for human and animal pituitary glands which is based on a combination of a modification of the phosphomolybdic acid, orange G stain of Berblinger and Burgdorf's (1935) method, with an iron hematoxylin stain for the nuclei and with the periodic-acid-Schiff routine of Hotchkiss and McManus. The cytochemical part of the procedure demonstrates both muco-protein precursors of the gonadotropins and, it is thought, these hormones themselves. By comparison with other methods, finer differences in the cytology of the cyanophils can be appreciated. Quantitative cell counts are readily performed and large numbers of cells, appearing by Mallory and other histological methods to be chromophobes, are found to belong to the cyanophil series. Counts done by older methods, therefore, are considered to be inaccurate and misleading.**

Professor J. R. Baker has said that the cytologist who can show a dye to be redundant generally deserves better of his fellows than he who introduces a new one, and the same may well be said of the introduction of a new method. Despite this, a new technic is here presented which has been used in studying the secretion of gonadotropin in human and rat pituitary glands, and which has been applied, with good cytological results, to the hypophyses of sheep and goats.

Rinaldini (1949), by biological assay, has shown that the pituitary glands of starved rats contain four times as much gonadotropin as those of normally fed animals. Pearse and Rinaldini (unpublished) using the periodic-acid-Schiff technic of Hotchkiss (1948) and McManus (1946, 1948a and b) have demonstrated that the glands from such starved rats contain a greatly increased amount of muco-protein. The HIO_4-Schiff technic was first used to demonstrate glyco-protein in the pituitary gland by Catchpole (1947) who described its association with the castrate cells in the rat and elaborated his findings in a later paper (1949). The method now described depends, first, on the ability of the HIO_4-Schiff routine to stain glyco- or muco-

proteins in the cells of the anterior lobe of the pituitary gland, secondly, on iron hematoxylin which is used to stain the nuclei and thirdly, on specific staining of the alpha granules of the acidophils by orange G.

Those substances have been considered to be mucoproteins which are demonstrable proteins by Millon's reaction and which give a strongly positive result with the HIO_4-Schiff method both after 30 minutes hydrolysis with malt diastase or 24 hours with testicular hyaluronidase. Additionally, they fail to give the characteristic metachromasia of the acid mucopolysaccharides and will not bind methylene blue from dilute (5×10^{-4} *M*) solutions below pH 4.0. Lipid substances are excluded by the application of Sudan black B to both frozen and paraffin control sections. By these methods, mucoprotein may be distinguished from polysaccharide (glycogen), glycolipid (cerebrosides), phospholipid (lecithin, cephalin), and acid mucopolysaccharide (mucin, hyaluronic acid); distinction from neutral mucopolysaccharide has not been achieved.

A large number of acid dyes have been investigated as counterstains for human and animal hypophyses following the application of the HIO_4-Schiff method. Those giving red shades of staining (eosins, phloxin, azocarmine) were rejected as giving insufficient contrast between cyanophils and acidophils. The blue and green acid dyes (aniline blue, fast green FCF, light green SF) were found to possess too little affinity for the alpha granules. Of the yellow dyes, picric acid, and orange G in neutral solution, both showed a similar lack of affinity. Below pH 4.9 orange G stained the alpha granules well and its combination with picric acid (picro-orange) also gave good results. In neither of these cases could the yellow stain be left in the alpha granules and washed out of the majority of other cytoplasmic structures. This effect was achieved by making use of the increased affinity of the alpha granules for both acid and basic dyes after mordanting wtih phosphotungstic acid. In combination with orange G the latter confers the necessary acidity on the solution and enables the counterstain to be washed out of all structures except the acidophil granules and red blood cells.

The following fixatives have been tried on human glands: formol saline, formol saline half saturated with mercuric chloride, Aoyama (Baker, 1945), Bouin, Carnoy, Helly, Zenker, 85% acetone and 80% alcohol. On the hypophysis of the rat, sheep and goat only formol saline, Helly, Carnoy and alcohol have been used. The method will give recognizable distinction between the three main types of cell in the pars anterior after each of these fixatives, except Carnoy, acetone

and alcohol; preservation of the specific granules, however, varies considerably. Good results are obtained even when the method is used after silver procedures (Aoyama) for demonstrating the Golgi apparatus. In man, and in those animals so far studied, the best results, with the clearest representation of the alpha granules of the acidophils and the mucroprotein granules of the cyanophils, are obtained after fixation in Helly's fluid containing 5% of neutralized formalin. Human and the larger animal glands are bisected in the horizontal plane with a sharp blade and the two halves are fixed and embedded separately; rat hypophyses are fixed *in situ*, after removal of the brain and overlying meninges, by immersing the whole base of the skull in a beaker of fixative.

The Trichrome-HIO_4-Schiff Procedure

1. Fix in Helly for 18 hours (man, goat, sheep) or 2–4 hours (rat).
2. Wash 6–12 hours in running water.
3. Dehydrate, clear and embed in paraffin wax.
4. Section at 4.5 μ (rat) or 5.5 μ (man).
5. Bring sections to water.
6. Remove mercury salts with Lugol's iodine solution, 3 min.
   (iodine, 1 g.; K.I., 2 g.; distilled water, 100 ml.) followed by 5% aqueous sodium thiosulfate, 3 min.
7. Wash in water.
8. Bring to 70% alcohol.
9. Immerse in Hotchkiss' periodic acid solution at room temperature, 5 min.

Periodic acid	0.4 g.
M/5 sodium acetate	5 ml.
Ethyl alcohol (pure reagent)	35 ml.
Distilled water	10 ml.

10. Flood with 70% alcohol.
11. Transfer to Hotchkiss' reducing rinse, 1 min.

Potassium iodide	1.0 g.
Sodium thiosulfate pentahydrate	1.0 g.
Ethyl alcohol (pure reagent)	30 ml.
Distilled water	20 ml.
2 *N* hydrochloric acid	0.5 ml.

12. Flood with 70% alcohol.
13. Transfer to Schiff's solution, 10–30 min.
    (De Tomasi's (1936) modification of Feulgen's stain has been used).

Basic fuchsin	0.5 g.
Dissolve in	
Boiling distilled water	100 ml.

Cool to 50° C. and add

N hydrochloric acid	10 ml.
Potassium metabisulfite	0.5 g.

Stand in the dark overnight.

Shake with 2 g. granulated charcoal for 1 min. to remove final traces of pink and filter before use.

14. Wash in running water, 10–30 min.

    (The sulfite baths of the Feulgen method appear to be unnecessary in routine use of this method).

15. Stain in celestin blue (Lendrum and Mcfarlane, 1940). ½ to 3 min.

Celestin blue (C.I. No. 900)	0.25 g.
Iron alum	2.5 g.
Distilled water	50 ml.

    Dissolve the alum in water, add celestin blue, boil for 3 minutes, filter when cool and add

Glycerol	7 ml.

16. Stain in Mayer's hemalum, ½ to 3 min.

Hematoxylin	0.1 g.
Sodium iodate	0.02 g.
Potassium alum	5 g.

    Dissolve overnight and add

Chloral hydrate	5 g.
Citric acid	0.1 g.

    Boil for 5 minutes and use when cool.

    Other iron hematoxylins can be used and 0.5% aqueous toluidine blue (C.I. No. 925) is a good alternative nuclear stain, especially after ribonuclease. If it is used in place of hemalum the following step is omitted.

17. Differentiate quickly in 2% acid alcohol.
18. Wash in running water, 5 min.
19. Stain in phosphotungstic acid–orange G solution, 10 sec.

Orange G (C.I. No. 27)	2 g.
Phosphotungstic acid, 5% aqueous	100 ml.

    Allow to stand for 48 hours, and use the supernatant.

20. Wash in water until a yellow tinge is just visible, 5–30 sec. Or control under the microscope.
21. Bring through alcohols to xylene and mount in clarite.

    No deterioration is visible after eighteen months unless an excess of phosphotungstic acid has been left in the section when some fading of nuclear staining is observed.

Discussion

Confirmation of the identity of the cyanophil granules with the HIO_4-Schiff-positive gamma granules has been obtained by first staining with the latter method and marking the red-stained granules in the cyanophils, preferably by photography. These are then de-

colorized by immersion in 0.5% potassium permanganate acidified with 0.15% of sulfuric acid, followed by 1% aqueous oxalic acid; the identical granules are then restained either with aniline blue or by means of basic dyes.

After the above staining, the beta granules in the cyanophils and a number of granules and vesicles in cells which stain as chromophobes by other methods are magenta to deep red; the colloid is magenta. The alpha granules of the acidophils are orange yellow and red blood cells a shade more yellow. The nuclei are blue black

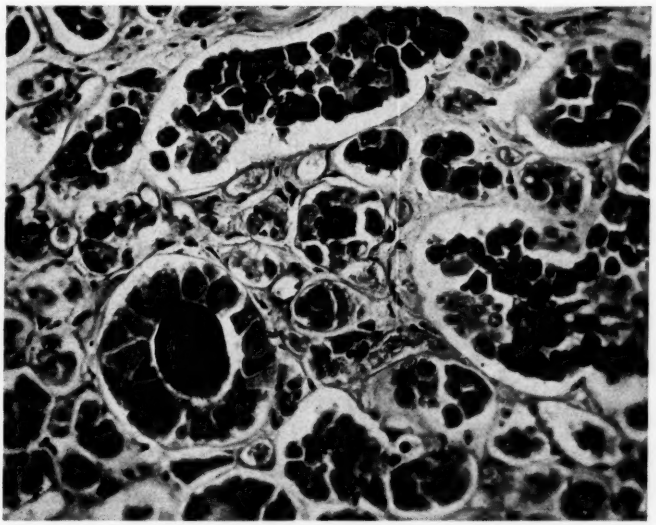

FIG. 1.—Human anterior hypophysis. Trichrome HIO_4-Schiff. ×250. Lower left, colloid in an acinus mainly composed of acidophils. Upper right, various cyanophils and a number of chromophobes containing HIO_4-Schiff-positive vesicles.

in color. If sections are treated, at stage 7, by immersion for up to one hour in a solution of ribonuclease, stages 15 and 16 can both be taken to fifteen minutes or more. This gives increasingly black nuclei and the gamma granules of the cyanophils remain free from additional staining by the hemalum. The word gamma is here used for all bodies composed of mucoprotein which are HIO_4-Schiff positive, and it includes, therefore, not only the beta granules but granules having no specific affinity for basic dyes or for the aniline blue of Mallory's method.

The ribonuclease used was prepared by the method of Kunitz (1940), dissolved to 0.05% in isotonic veronal acetate buffer at pH 6.75 and kept at room temperature. It is unnecessary to use ribonuclease as a routine procedure. In the writer's experience it has proved that aqueous solutions may be employed in place of Hotchkiss' alcoholic ones. The theoretical conception that the mucoprotein-periodic-acid reaction product may be water soluble renders the use of alcoholic solutions necessary if histochemical conclusions are being drawn; the mucoprotein granules are still completely

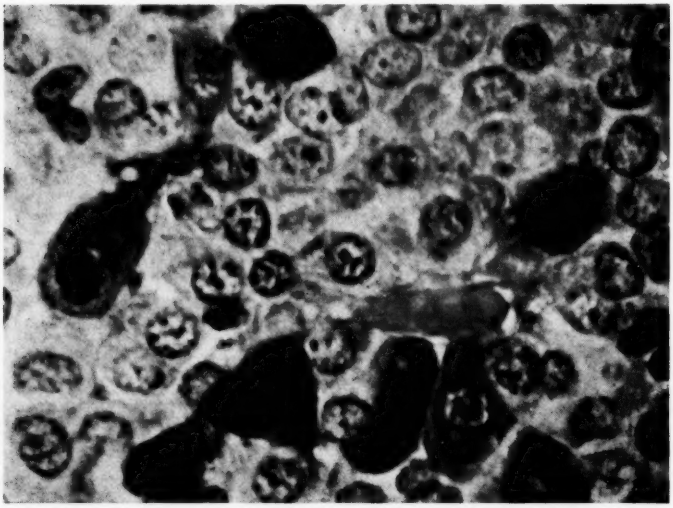

FIG. 2.—Rat anterior hypophysis. Male, 26 months. Trichrome HIO_4-Schiff. ×1050. Several dark staining cyanophils (gamma cells), some containing irregular masses of mucoprotein, are present. In the top left hand corner are two chromophobes and another is visible at lower right. The cells above the blood vessel (right center) are nearly all acidophils.

water soluble after alcoholic fixation and possibly after other fixatives, formalin seems to render them entirely insoluble.

Fig. 1 is from a 5 μ section of the hypophysis of a 74-year-old woman, stained with trichrome HIO_4-Schiff; good differentiation between the cell types can be seen even in this black and white photograph. It will be noticed that few chromophobes are present and that of these the majority contain dark (red) staining granules in a pale background. These bodies occur in so-called chromophobes in all species so far examined, their frequency being greatest

in the human gland where they appear as vesicles rather than granules. Pearse (1948, 1949) considers that these vesiculate chromophobes belong to the cyanophil series. Modified Rasmussen counts, after the manner of Halpern (1938), performed on human hypophyses stained by trichrome HIO_4-Schiff have shown that the true percentage of cyanophils is from 12–25% higher than when estimated in Mallory-stained serial sections (Pearse, unpublished). If it is accepted that a number of cells formerly classified as chromophobes belong, in fact, to the cyanophil series, it follows that percentage cell counts of human glands using Mallory's stain, or in the rat using methods such as those of Severinghaus (1932), Cleveland and Wolfe (1932), Mellgren (1944), or the most recent method of Briseno-Castrejon and Finerty (1949), will be inaccurate. Moreover, they all fail to give an accurate picture of the state of gonadotropin production and storage in the gland.

Fig. 2 is a photomicrograph of the hypophysis of an old male rat. This illustrates the efficiency of the trichrome-HIO_4-Schiff method for revealing finer differences in mucoprotein content of the cyanophils in the various stages of their secretory cycle. Although distinction between chromophobes and acidophils is not clearly shown in the photograph, differentiation between all three cell types is very clear under direct vision, preferably using a blue filter. Quantitative cell counting is thus easy to perform but projection methods cannot be applied.

REFERENCES

Baker, J. R. 1945. Cytological Technique. Methuen, London, p. 101.

Berblinger, W., and Burgdorf, A. L. 1935. Neue Farbemethode zur Darstellung der Gewebsbestandteile der Hypophyse des menschen. Endokrinologie, **15**, 381–8.

Briseno-Castrejon, B., and Finerty, J. C. 1949. An azocarmine stain for differential cell analysis of the rat anterior hypophysis. Stain Techn., **24**, 103–7.

Catchpole, H. R. 1947. Cellular distribution of glycoprotein in the anterior lobe of the pituitary gland (abstract). Federation Proc., **6**, 88.

———— 1949. Distribution of glycoprotein hormones in the anterior pituitary gland of the rat. J. Endocr. **6**, 218–25.

Cleveland, R., and Wolfe, J. M. 1932. A differential stain for the anterior lobe of the hypophysis. Anat. Rec., **51**, 409–13.

De Tomasi, J. A. 1936. Improving the technic of the Feulgen stain. Stain Techn., **11**, 137–44.

Halpern, S. R. 1938. Quantitative cytological studies of the anterior lobe of the hypophysis of fetuses and children, correlated with sexual and skeletal development. Endocrinology, **22**, 173–80.

Hotchkiss, R. D. 1948. A microchemical reaction resulting in the staining of polysaccharide structures in fixed tissue preparations. Arch. Biochem., **16**, 131–41.

KUNITZ, M. 1940. Crystalline ribonuclease. J. Gen. Physiol., **24,** 15–32.

LENDRUM, A. C., and MCFARLANE, D. 1940. A controllable modification of Mallory's trichromic staining method. J. Path. Bact., **50,** 381–4.

MCMANUS, J. F. A. 1946. Histological demonstration of mucin after periodic acid. Nature, **158,** 202.

MCMANUS, J. F. A. 1948a. The periodic acid routine applied to the kidney. Amer. J. Path., **24,** 643–53.

MCMANUS, J. F. A. 1948b. Histological and histochemical uses of periodic acid. Stain Techn., **23,** 99–108.

MELLGREN, J. 1944. A differential stain for the anterior lobe of the hypophysis of rats and mice. Acta path. Scand. Suppl., 54.

PEARSE, A. G. E. 1948. Cytochemistry of the gonadotropic hormones. Nature, **162,** 651.

PEARSE, A. G. E. 1949. The cytochemical demonstration of gonadotropic hormone in the human anterior hypophysis. J. Path. Bact., **61,** 195–202.

RINALDINI, L. M. 1949. Effect of chronic inanition on the gonadotrophic content of the pituitary gland. J. Endoc., **6,** 54–62.

SEVERINGHAUS, A. E. 1932. Cytological technique for the study of the anterior lobe of the hypophysis. Anat. Rec., **53,** 1–5.

THE MECHANISM OF THE GRAM REACTION
I. THE SPECIFICITY OF THE PRIMARY DYE

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ABSTRACT.—**Dyes of all major types were tested for their suitability as the primary dye in the Gram stain. When a counterstain was not used, some dyes of all types were found to differentiate Gram-positive from Gram-negative organisms. When a counterstain was used, these dyes were found to vary greatly in their suitability. Those dyes found to be good substitutes for crystal violet were: Brilliant green, malachite green, basic fuchsin, ethyl violet, Hoffmann's violet, methyl violet B, and Victoria blue R. All are basic triphenylmethane dyes. Acid dyes were generally not suitable. Differences in the reaction of Gram-positive and Gram-negative cells to Gram staining without the use of iodine were observed and discussed but a practical differentiation could not be achieved in this manner. Certain broad aspects of the chemical mechanism of dyes in the gram stain are discussed.**

Conflicting statements are found in the literature regarding the ability of various dyes to serve as the primary stain in the Gram reaction. Burke and Barnes (1929) stated that the Gram reaction will not occur unless the primary dye forms a precipitate with iodine. This precipitate must be insoluble in water and soluble in the decolorizer. Knaysi (1946) stated that the methyl violets were best, Victoria blue and basic fuchsin successful, whereas methylene blue was found to be unsuitable. Bartholomew and Umbreit (1944) stated that some part of the structure of crystal violet is specific. The present survey was undertaken in an effort to clarify this aspect of the Gram stain mechanism. It should be stated that this work is not an attempt to develop a substitute for crystal violet, but is part of a broader program aimed at elucidation of the mechanism of the Gram stain.

METHOD

Dyes were made up in 0.5% solution as follows: 0.25 g. dye was dissolved in 1 ml. of 95% ethyl alcohol and diluted to 50 ml. with distilled water. The pH was then adjusted to near neutrality. An

TABLE 1. ABILITY OF VARIOUS DYES TO TAKE PART IN THE GRAM REACTION (NO COUNTERSTAIN)

Dye	Acid or base	Gram reaction	Ppt. with iodine	Dye data
DIPHENYLMETHANES				
Auramine O	b	+	+	Cert. No. NAu–2
TRIPHENYLMETHANES (DIAMINO)				
Brilliant green	b	+	+	Grübler
Light green SF yellowish	a	—	—	Cert. No. NL–10 (Nat. Aniline)
Fast green	a	—	—	Cert. No. NGf–9
Malachite green	b	+	+	Cert. No. NMg–13 (Nat. Aniline)
TRIPHENYLMETHANES (TRIAMINO)				
Crystal violet	b	+	+	Cert. No. NC-32 (Nat. Aniline)
Basic fuchsin	b	+	+	Cert. No. NF–54 (Nat. Aniline)
New fuchsin	b	+	+	Nat. Aniline–12096
Acid fuchsin	a	+	+	Coleman & Bell—420812
Carbol-fuchsin	b	+	+	Cert. No. NF–54 (Nat. Aniline)
Ethyl violet	b	+	+	Nat. Aniline—8673
Hoffmann's violet	b	+	+	Nat. Aniline—10799
Methyl violet B	b	+	+	Unknown
Methyl green	b	+	+	Cert. No. NG–20 (Nat. Aniline)
Victoria blue B	b	+	+	Nat. Aniline—10098
Victoria blue R	b	+	+	Nat. Aniline—10110
Victoria blue 4R	b	+	+	Nat. Aniline—12802
Aniline blue WS	a	+	+	Cert. No. NK–9
Pararosaniline	b	+	+	Paragon Testing Lab.—5693
Acid violet	a	—	—	Nat. Aniline—12787
AZO DYES (MONOAZO)				
Janus green B	b	+	+	Cert. No. NJ–2 (Nat. Aniline)
Chrysoidin Y	b	+	+	Unknown
Orange G	a	—	—	Nat. Aniline (no number)
AZO DYES (DIAZO)				
Bismarck brown Y	b	+	+	Cert. No. NN–4 (Nat. Aniline)
Congo red	a	—	—	Baker—S71229
QUINONEIMINES (INDAMINS)				
Bindschedler's green	b	+	+	Nat. Aniline—12254
QUINONEIMINES (INDOPHENOLS)				
Sodium benzeneone-indophenol	a?	—	—	Nat. Aniline—11311
QUINONEIMINES (THIAZINS)				
Methylene blue	b	+	+	Cert. No. NA–27 (Nat. Aniline)
Methylene violet	a?	—	—	Coleman & Bell (no number)
Azure II	b	+	+	Nat. Aniline—5982
Toluidine blue O	b	+	+	Cert. No. NU–1 (Nat. Aniline)
Thionin	b	+	+	Cert. No. NT–14 (Nat. Aniline)

TABLE 1. *Concluded*

Dye	Acid or base	Gram reaction	Ppt. with iodine	Dye data
QUINONEIMINES (OXAZINS)				
Cresyl violet	b	+	+	Cert. No. NW–17
QUINONEIMINES (AZINS)				
Neutral red	b	+	+	Cert. No. NX–9 (Nat. Aniline)
Safranin Y	b	+	+	Cert. No. CS–11 (Coleman & Bell)
Nigrosin	?	—	—	Cert. No. NNi–8 (Nat. Aniline)
XANTHENES				
Pyronin Y	b	+	+	Cert. No. NP–10 (Nat. Aniline)
Rhodamine B	b	+	+	Nat. Aniline—11534
Eosin Y	a	—	—	Cert. No. NE–13 (Nat. Aniline)
Brom thymol blue	a	—	—	Difco—388475
Brom cresol purple	a	—	—	Difco—385050
Acriflavine	b	+	+	Abbott—539126
Rose bengal	a	—	—	Unknown

effort was made to include at least one representative of each type of dye used in bacteriological staining. Many of the dyes were obtained through the courtesy of Dr. H. J. Conn.

The test organisms were *Bacillus subtilis, Neisseria catarrhalis,* and *Escherichia coli,* grown on nutrient agar slants at 37° C. for 18 hours. The cells were suspended in distilled water, the turbidity adjusted to approximately that of a No. 8 barium sulfate standard, and a loopful (calibrated to contain 0.01 ml.) spread over approximately 1 sq. cm. on the slides. This procedure gave thin uniform smears. One smear of each of the three organisms was placed on a slide.

Two modifications of the Gram stain were used: (1) The Hucker-Conn (1923) method, following the procedure and using reagents as given in Diagnostic Procedures and Reagents (1945), but omitting the ammonium oxalate. This technic involves staining at about pH 6.4. (2) The Kopeloff-Beerman (1922) method. In this modification 5% $NaHCO_3$ is added, raising the pH of the primary stain to about 9.3. The iodine solution is also alkaline. Both methods gave essentially the same results, but the Kopeloff procedure generally resulted in a more intense Gram positivity. All results recorded represent at least four trials of the dye. In some cases where results were questionable the reported results represent our opinion after twelve different trials.

Safranin or methylene blue in 0.25% solutions were used as counterstains, depending on the color of the primary stain.

Results

A. NO COUNTERSTAIN

The Gram stain was applied first without using a counterstain. This was done to detect the dyes which would function in the chemical reaction involving dye, iodine, and cell material. The counterstain was omitted to avoid masking any positive reaction which might occur with a dye of low color value. These results are given in Table 1. A plus sign for the Gram reaction indicates that the dye was retained after decolorization by the Gram-positive cells but not by the Gram-negative cells (i.e., it undergoes a reaction similar to that of crystal violet). A minus sign indicates that the dye was retained by neither Gram-positive nor Gram-negative cells. A plus sign for precipitation with iodine indicates that the dye formed a water-insoluble precipitate. The acid or basic nature of the dye is indicated.

Two interesting correlations were observed: (1) The dye which yielded a precipitate with iodine always gave a Gram differentiation. (2) In general, the basic dyes gave differentiation, while the acid dyes did not.

The great variability of the dyes in their rates of decolorization by alcohol is not shown in Table 1. Some dyes resisted decolorization very tenaciously while some were decolorized almost immediately. In all cases where a positive reaction is noted, the Gram-negative cells were the first to decolorize.

B. WITH COUNTERSTAIN

The staining procedure was repeated. This time a counterstain was applied. On this basis the dyes were divided into three groups, as indicated in Table 2. The first column lists those which gave excellent differentiation, comparable to crystal violet (although none quite equalled the clarity and brilliance of crystal violet.) The second column contains those dyes which differentiated Gram positives from Gram negatives but in an inferior manner, due to incomplete masking, displacement, poor staining, contrasting qualities, or irregular staining. The third column lists those dyes which gave no differentiation, either due to masking of the primary stain by the counterstain, or the displacement of the primary stain by the counterstain (Bartholomew, Evans, and Roberts. Forthcoming publication). The counterstain procedure considerably reduced the number of usable dyes as primary stains in the Gram reaction.

C. STAINING WITHOUT IODINE

In a study of mordants, to be reported later, preliminary experiments were carried out with various dyes using no iodine but otherwise using normal Gram-staining procedure. In all cases tried, the *E. coli* was decolorized first, *N. catarrhalis* second, and *B. subtilis* last, exactly the order of decolorization in a regular Gram stain. In all cases the decolorization time was comparatively short and great care had to be exercised to prevent overdecolorization. Counterstains applied to cells stained without iodine completely replaced the primary dye in Gram-positive cells in less than a minute. Thus,

TABLE 2. ABILITY OF VARIOUS DYES TO TAKE PART IN THE GRAM REACTION (WITH COUNTERSTAIN)

Excellent differentiation	Inferior differentiation	No differentiation
Brilliant green	Auramine O	*Aniline blue* WS
Malachite green	New fuchsin	*Methylene violet*
Crystal violet	*Acid fuchsin*	Rhodamine B
Basic fuchsin	Carbol fuchsin	*Light green*
Ethyl violet	Methyl green	*Fast green* FCF
Hoffmann's violet	Victoria blue B	*Acid violet*
Methyl violet B	Victoria blue 4R	*Orange G*
Victoria blue R	Pararosaniline	*Congo red*
	Janus green B	*Sodium benzeneone-indophenol*
	Chrysoidin Y	*Nigrosin*
	Bismarck brown Y	*Eosin* Y
	Bindschedler's green	*Brom thymol blue*
	Methylene blue	*Brom cresol purple*
	Azure II	*Rose bengal*
	Toluidine blue O	
	Thionin	
	Cresyl violet	
	Neutral red	
	Safranin Y	
	Pyronin Y	
	Acriflavine	

NOTE: Italicized dyes are acidic or weakly basic. Non-italicized dyes are basic.

to achieve Gram differentiation, counterstaining time had to be drastically reduced and carefully controlled. Foreknowledge of the Gram nature of the organisms in this experiment made their Gram differentiation much easier. We in no way mean to imply that iodine should be omitted from the Gram staining procedure.

DISCUSSION

The basic dyes all gave differentiation when the counterstain was omitted. When a counterstain was used all basic dyes gave good or inferior differentiation except rhodamine B. This latter result was due to the light staining characteristics of this dye under the condi-

tions of these experiments. The staining was so light that the counterstain completely masked it. The acid dyes gave no differentiation except for acid fuchsin and aniline blue WS. The activity of acid fuchsin is hard to explain. However, acid fuchsin is a triphenylmethane dye, and all of the good primary dyes for gram differentiation are basic members of this group. According to Conn (1940) the acid fuchsins on the market are usually a complex mixture composed of several of the twelve possible structurally different acid fuchsins. Aniline blue WS and acid violet are often similar mixtures but phenylated, and thus of higher molecular weight than acid fuchsin, especially the particular acid violet used in this study (C.I. No. 698) which is a tetraethtyl-triphenyl derivative. Because of this similarity, it is strange the three dyes should behave so differently: the one of lowest molecular weight (acid fuchsin) giving differentiation either with or without a counterstain; the one of intermediate weight (aniline blue) showing differentiation with no counterstain but none with the counterstaining technic followed in this work; while the heaviest (acid violet, C.I. No. 698) showed no differentiation even in the absence of a counterstain—thus behaving like the majority of acid dyes. It is noteworthy that Scales (1922) used some variety of aniline blue to develop a new method for Gram differentiation of bacteria. His method omitted both the iodine and decolorization step. Thus aniline blue WS has truly differential properties, although weak and unsuitable for the usual Gram procedure.

The foregoing data also show that some dyes of all major types undergo a reaction similar to that of the classic Gram stain when no counterstain is used.

Burke and Barnes (1929) gave the name "pseudo Gram reaction" to instances where the first dye was retained but subsequently masked by a counterstain, if used. They believed that in the "pseudo Gram reaction" there was a union of dye and protoplasm, but the protoplasm was not heavily stained. Thus, if a counterstain was then applied (the "true Gram reaction"), the first dye would be masked.

We are in agreement with these authors concerning the essential nature of the counterstain in a true Gram reaction. Our differentiation in the absence of iodine is even further from a true Gram reaction. We are now conducting experiments to determine whether differentiation without iodine is part of the true Gram reaction, or entirely independent of it.

Although Burke and Barnes were discussing the role of the

"mordant" in their statement that precipitate formation between dye and iodine was necessary for the Gram reaction, the implication was that any dye and any "mordant" would function if the proper precipitate was formed. The data here presented tend to confirm this observation insofar as dyes and iodine are concerned. All dyes which gave a positive Gram reaction also formed a precipitate with iodine. Conversely, those dyes which did not form a precipitate with iodine did not give a Gram stain. However, Burke and Barnes interpret their results as indicating the formation of large dye-iodine molecules which could not pass out through the cell membrane.

We do not agree entirely with this interpretation. Subsequent papers in this series will show that the reason for the differential retention of the dye-iodine precipitate is a matter of differences in cell permeability to alcohol, and to iodine in alcoholic solution. We will also present evidence to show that chemical factors also operate in the Gram stain, by influencing permeability and by the formation of a Gram positive complex.

Table 2 shows that eight of the dyes tested gave good usable Gram stains. These were all triphenylmethanes. It is possible that some of the other dyes could be made to "work" in the Gram stain by varying concentrations and times in the various steps. Thus, a re-study of the Gram technic would be necessary if crystal violet became unavailable and the necessity arose to substitute one of the other dyes listed as possibilities in Table 2.

Acknowledgment

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BIBLIOGRAPHY

American Public Health Association. 1945. Diagnostic Procedures and Reagents, *2nd Ed.* A.P.H.A., New York. p. 46.

Bartholomew, J. W., and Umbreit, W. W. 1944. Ribonucleic acid and the gram stain. J. Bact., **48,** 567.

Burke, V., and Barnes, M. W. 1929. The cell wall and the gram reaction. J. Bact., **18,** 69.

Conn, H. J. 1940. Biological Stains. *5th Ed.* Biotech. Publs., Inc., Geneva, N. Y.

Hucker, G. J., and Conn, H. J. 1923. Methods of gram staining. N. Y. (Geneva) Agr. Exp. Sta. Tech. Bull. 93.

Knaysi, G. 1946. Elements of Bacterial Cytology. Comstock Publishing Co., Ithaca, N. Y.

KOPELOFF, N., and BEERMAN, P. 1922. Modified gram stains. J. Inf. Dis., 31, 480.

SCALES, F. M. 1922. A new method for differential staining of bacteria. J. Inf. Dis., 31, 494.

NOTES ON TECHNIC

The Use of Oxalic Acid in Staining with Phloxine and Hematoxylin[1]

Since the introduction of phloxine as a counterstain by Mallory, the use of this agent in staining technics has been largely confined to the staining of tissues fixed with Zenker's solution with methylene blue as a nuclear stain. In a recent article Rosenbaum[2] has discussed the use of phloxine as a counterstain with hematoxylin for histologic use, and has outlined his technic.

The purpose of this report is to present the results of using oxalic acid in the phloxine bath. Considerable experimental staining was carried out over a period of several years to determine the most satisfactory technic for the use of phloxine as a counterstain. It was discovered that the addition of a small amount of oxalic acid to the phloxine had an enhancing effect on the quality of the cytoplasmic stain. The result was not only a definite improvement in the quality and uniformity of the staining, but also a shortened staining process and a decreased amount of stain needed for results comparable to those obtained with unacidified aqueous phloxine.

A standard technic using 1% solution of phloxine in 0.05% aqueous oxalic acid (5 ml. of 1% oxalic acid for each 100 ml. of 1% aqueous stain is convenient) was tentatively established by trial and error. This is a change from approximately pH 8 for unacidified 1% aqueous phloxine to approximately pH 6 for the acidified stain. Subsequently, a series of staining experiments were conducted using this modified technic in comparison with other technics, including Wadsworth's hematoxylin-phloxine technic.[3] In these studies the tissues stained were all chicken tissues, but equally satisfactory results have been obtained over a period of several years with tissues from various mammals. Slides of both avian and mammalian tissues have shown no fading after several years. Furthermore, this staining technic has proved to be equally satisfactory following fixation with Bouin's fixative, 10% formalin, or Zenker's solution,

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[1]Published as Journal Paper No. 381 of the Purdue University Agricultural Experiment Station.

[2] Rosenbaum, R. 1947. Phloxine as an histologic stain, especially in combination with hematoxylin. Stain Techn., **22**, 149–153.

[3]Wadsworth, A. B. 1939. Standard methods of the Div. of Lab. and Res. of the N. Y. State Dept. of Health, *2nd Ed.* Williams and Wilkins, Baltimore.

and with either ethyl alcohol, butyl alcohol, or carbon disulfide methods of dehydration.

From these tests, which involved 3400 test slides and 17 variations in staining technic, it was determined that 1) acidification with either oxalic acid or acetic acid has an enhancing effect on phloxine, but oxalic acid is easier to control and gives better differentiation; 2) acidification with oxalic acid has a similar effect on other counterstains, such as orange G and eosin Y; and that 3) our acidified phloxine technic proved to be most satisfactory and convenient for routine use.—ARTHUR L. DELEZ and OLIVE STULL DAVIS, *Lafayette, Ind.*

CEDARWOOD OIL AS A CLEARING AGENT WITH ACETIC-ALCOHOL FIXATIVES

For some time microtomists have been aware that cedarwood oil, while in use as a clearing agent, will occasionally undergo a change involving the formation of a gelatinous mass of needle-like crystals which eventually renders this expensive reagent useless. Lillie[1] has drawn attention to a correlation between the appearance of this phenomenon and the use of acetic-alcohol fixatives. However, this does not seem to be a simple one-to-one cause and effect relationship. In order to test the action of these mixtures as impurities, sixteen bottles were carefully cleaned and 12 ml. of high quality unused cedarwood oil added to each. To one series of four bottles amounts of $^1/_{60}$, $^1/_{40}$, $^1/_{20}$, and $^1/_{10}$ ml. of both absolute ethyl alcohol and glacial acetic acid were introduced. To another series of four, the same amounts were added using Carnoy's acetic-alcohol mixture (Lee's microtomists' Vademecum, *10 Ed.*, Sec. 90). To a third series Zenker's fixative (Id., Sect. 78) was employed with corresponding amounts of alcohol, and finally, the Carnoy-Lebrun mixture (Id., Sect. 91) which is relatively unstable producing ethyl acetate in a short time. These were left at room temperature (26° C.) for 24 hours, then placed in a refrigerator at 10° C. The characteristic crystalline mass did not appear in any of the mixtures. It seems most probable, therefore, that the reaction is dependent upon the presence of certain tissue substances in addition to traces of acetic acid and alcohol.

Once initiated, however, this formation will grow at the expense of the liquid cedarwood oil in the absence of tissues. Moreover, although the melting point of this material is 35° C., the optimum

[1]Lillie, R. D. 1948. Histopathologic Technic. Blakiston Co., Philadelphia.

temperature for its development is below 26° C. When it appeared in an incipient stage in two bottles containing guinea pig thyroids in 40 ml. of the oil, the tissues were removed and the cedarwood oil left for 24 hours at room temperature (26° C.). Under these circumstances the precipitate settled out in one and failed to develop further in the other. Upon cooling both to 10° C. for 12 hours, a fully developed crystalline formation involving the entire 40 ml. of cedarwood oil appeared.

It remains to point out that the addition of as little as 0.5 ml. of xylene to 40 ml. of cedarwood oil was found to prevent the formation of this substance and to dissolve it once formed. This is probably the result of the action of the xylene upon the alcoholic component of the impurities responsible for the crystalline precipitate. Lillie (l. c.) suggests heating the oil to 200° C. to melt the crystals and boil off impurities. However, the addition of a little xylene, which is itself a good clearing agent and quite miscible, seems to be entirely satisfactory for most purposes and has not been observed to interfere in any way with the effectiveness of the cedarwood oil as a clearing agent.—Robert E. Popham, *Faculty of Medicine, Univ. of Toronto, Toronto, Canada.*

An Improved Embedding Box For Paraffin

A method is presented for preparing containers for making paraffin blocks that offers considerable advantage in speed and ease over the common methods of folded-paper boxes, plaster-of-Paris boxes, watch glasses, etc. The light weight aluminum foil now sold for kitchen use is simply pressed by the fingers about a suitable mold. It is convenient to glue to a mounting board a number of wooden blocks of sizes such as are routinely used in the individual laboratory. Overlap of the foil onto the board provides a convenient flange for handling. Reasonable care must be taken not to tear the foil upon corners of the blocks. Fit does not need to be precise, for the fingers can easily mold the foil to the exact shape of the block. Aluminum is a good conductor of heat, so a basal layer of solidified paraffin is quickly obtained by partial immersion of the container in cold water. After the block has hardened, the foil is easily removed and often can be re-used, although the foil is inexpensive. The technic is useful when embedding in "Carbowax" but should not be used for celloidin, as the vapors do not penetrate the foil to produce hardening.—E. Russell Hayes and Nancy P. Hayes, *Department of Anatomy, The Ohio State University, Columbus, O.*

Rapid Staining of Nematodes for Taxonomic Studies[1]

Many methods of staining Nematodes have been employed by different investigators, some of which methods are very complex and time consuming. The present technic is time-saving, inexpensive and very convenient. Animals stained by this method can be used in taxonomic work and may be stored without fear of discoloration.

Round worms for *in toto* preparations are washed in the appropriate saline solution, then transferred to a small vial containing fresh saline. When the worms are ready for fixing, most of the saline is removed with the aid of a dropper. Carnoy's fluid (6:3:1) is added immediately, and the vial is shaken vigorously for a few seconds (Looss' method) before corking.

A piece of Eye-Ease Hammermill Filler paper[2] of about 2 to 3 square inches is inserted in the vial until covered by the fixative. The dyes employed in the manufacture of this paper go readily into solution and are taken up by the tissues of the worms. The intensity of the stain can be controlled by replacement of the bleached paper by a new one if a darker staining is desired, or by partial replacement of the fluid if too dark.

After being allowed to stand overnight, the Carnoy's fluid is replaced by 95% ethanol. The specimens are hydrated gradually, leaving them 10 minutes in each of 70%, 50%, and 30% ethanols, and finally putting them into distilled water. Individual worms are mounted in Yetwin's medium.[3] Slides can be made permanent by sealing the cover glass with piccolyte or balsam after the medium has hardened. The procedure is:

(1) Fix in Carnoy's fluid (6:3:1)
    Absolute alcohol ..... 6 parts
    Chloroform ..... 3 parts
    Glacial acetic acid ..... 1 part
(2) Insert a piece of Eye-Ease Hammermill Filler paper into the vial. Let stand overnight.
(3) Hydrate in alcohols to water
    95%, 80%, 70%, 50%, 30% .....(10 min. each)
(4) Mount in Yetwin's medium

—Honorico Ciordia, *Department of Zoology and Entomology, University of Tennessee, Knoxville, Tenn.*, and Nettie Cate Johnston, *Hollins College.*

[1]Contribution from the Dept. of Zoology and Entomology, Univ. of Tennessee, Number 35.

[2]This paper is made by Hammermill Paper Co., Erie, Pa. and is sold by National Blank Book Co., Holyoke, Mass. By communication with the manufacturer, it has been learned that the following dyes are used in making this paper: burnt turkey umber, anthroquinone blue BN, soluble blue PB–6–D, and brilliant paper yellow ex. cone.

[3]Yetwin, I. J. 1944. A simple permanent mounting method for *Necator americanus*. J. Parasitol., **30**, 201.

LABORATORY HINTS
FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

BOOK REVIEWS

DUBREUIL, GEORGES, and BAUDRIMONT, ALBERT. **Manuel Théorique et Pratique d' Histologie.** *Vigot, Paris.* 1950. 635 pages (730 figures). 21 x 27 cm. Price 3000 francs.

The 4th edition of this internationally known text summarizes in the first 24 pages the technics whereby the functions of a tissue may be studied in comparison with its histological and cytological structures; pages 27–48 present a critical study of "general cytology" and prescribe the most efficient technics for the demonstration of nuclear constituents and cytoplasmic enclosures, notably mitochondria, and vacuolar material.

The 600 pages of text, illustrated with 730 figures, discuss the histology of the various tissues, as demonstrated by the use of the proper stain technology.—*J. Dufrenoy.*

ROULET, F. **Methoden der pathologischen Histologie.** 6½ x 9½ inches. 567 pages. Cloth. *Springer Verlag. Vienna.* 1948. Price $16.00.

This book was written as a substitute for Schmorl's well known work: "Die pathologisch-histologischen Untersuchungsmethoden" and follows more or less the same plan. For a treatise on microscopic technic it has the serious shortcoming that it contains many inaccuracies. Thus in the description of Petersen's method using acid alizarin blue and aniline blue (p.285) the differentiation in phosphotungstic acid had been omitted; the formula for Weigert's glia mordant contains copper sulfate instead of copper acetate (p.421) and in Lison's method for the staining of hemoglobin the concentration of the H_2O_2 is not stated (p.242). Although it is stated that Bouin's fluid with trichloracetic acid is to be preferred for the fixation of cytologic details (p.46), the formula on p.46 and p.53 contains acetic acid, a mixture which was discarded many years ago by Masson, Foot and others. The reviewer misses such practical hints as the collodionization of sections which are to be stained with strongly alkaline or acid solutions or the neutralizing of formaldehyde solutions by buffering instead of by the inadequate calcium carbonate method.

With one exception (Highman's method for the staining of amyloid) the publications on microscopic technic which have appeared in the American and English literature after 1939 are completely ignored. Therefore, the inexperienced reader gets a very incomplete impression of the methods, stains, mounting media and apparatus available or of the research on stains and microscopic technic that has been going on since the beginning of the second World War. There is nowhere any reference to the Colour-Index or to the work of the Biological Stain Commission.

In addition to the stains produced by Swiss and French manufacturers the author recommends the stains of Hollborn, although it has been shown that several important German stains may be unsatisfactory (Stain Techn., **24**, 1, 1949).

The technics used in the laboratories of continental Europe, especially in those of Switzerland, France and Germany, are exhaustively described. Thus the book contains several useful methods with which workers in America may not be conversant such as Held's and Clara's molybdic acid hematoxylin methods, Heidenhain's picro-blue-black-B stain for connective tissue, Ferner's method for the impregnation of the alpha-cells of the islets of Langerhans with silver, the excellent though laborious method of Weber for the impregnation of neurofibrils, Romeis' new technic for the differential staining of the cells of the anterior lobe of the hypophysis and Hallberg's night blue stain for acid fast bacilli.

For the Mallory-Masson methods, the author recommends Wasserblau 2108/A and Tintenblau H, both manufactured by Geigy, instead of aniline blue.

The American pathologist, who is interested in and well versed in histologic technic, will read this book with profit; but by reason of its incompleteness and its inaccuracies it is not to be relied on exclusively and is certainly not to be recommended for beginners.—*Ph. H. Hartz.*

MICROSCOPE AND OTHER APPARATUS

BAKER, J. R. **A new method for oblique microscopical illumination.** *Quart. J. Micr. Sci.*, **89**, 233–8. 1948.

The purpose of the method is to facilitate the resolution of extremely fine regularly repeated markings on microscopical objects. Resolution is obtained by using the objective as an eyepiece to observe the *direction* and *degree* of obliquity of the light; these are controlled by movements of the mirror. To enable the objective to be used in this way, the condenser is raised above the position required for so-called "critical" illumination. The diffraction-spectrum formed by the markings on the object is clearly seen while the mirror movements are being made and it is easy to produce a bright and complete spectrum.

RICHARDS, O. W. **Some recent advances in microscopy.** *Trans. Amer. Micr. Soc.*, **68**, 292–303. 1949.

This is a brief summary of postwar improvements and new developments in microscopes, lighting, accessories, types of microscopy, and microtoming.—*V. Kavanagh.*

THOMAS, O. L. **Some observations with phase-contrast microscope on the neurones of *Helix aspersa*.** *Quart. J. Micr. Sci.*, **88**, 269–73. 1947.

The phase-contrast microscope reveals the presence of neurofibrils within the freshly teased neurons of *Helix aspersa*. Each fibril resembles a row of minute cocci. Individual fibrils appear to pass from axon to cell body, loop round the nucleus and, retaining their identity, pass back into the axon. No evidence of lateral anastamoses nor network formation of the fibrils could be found.

PHOTOMICROGRAPHY

LOVELAND, R. P. **Color photomicrography in the laboratory.** *Anal. Chem.*, **21**, 467–75. 1949.

Use of a roll-film camera and flash bulbs makes darkroom and processing facilities unnecessary. It is advantageous to have an image plane that is open for inspection and analysis, and in which the film is placed directly. Methods for the quantitative and qualitative analysis of illumination are described and criteria of optimum exposure are given.—*R. T. Whittenberger.*

MICROTECHNIC IN GENERAL

BROOKE, M. M., and GOLDMAN, MORRIS. **Polyvinyl alcohol fixative as a preservative and adhesive for protozoa in dysenteric stools and other liquid materials.** *J. Lab. & Clin. Med.*, **34**, 1554–6. 1949.

The authors recommend a fixative prepared by adding 5.0 g. polyvinyl alcohol to a mixture of 4.5 ml. glycerol, 5.0 ml. glacial acetic acid and 93.5 ml. of a mixture of 2 vol. of saturated aqueous $HgCl_2$ with 1 vol. 95% ethanol. The following procedure is recommended: Place one drop of material on a slide with an applicator stick; add 3 drops of fixative; wash and stain by any method desired. This fixative acts both as a fixing fluid and adhesive.—*John T. Myers.*

COBURN, W. H. **Aerosol as an aid to bone decalcification.** *Bul. Intern. Assoc. Med. Museums*, **29**, 93. 1949.

The author has tried the use of wetting agents to cut down the time necessary to decalcify bone specimens and found best results with aerosol (dioctyl sodium sulfosuccinate). Specimens fixed in Zenker's fluid and washed 4–6 hours are placed in the following solution: HNO_3 (conc.?), 5 ml.; 10% aqueous aerosol, 1 ml.; water (distilled?), 100 ml. (The HNO_3 solution can be kept on hand, but the aerosol must be added to it just before use.) Decalcification requires 4 hr. or less for small specimens, but the larger pieces need overnight treatment. Formalin-fixed tissues may be treated similarly, but results are less satisfactory.—*H. J. Conn.*

DYES AND THEIR BIOLOGICAL USES

KENDALL, J. D. **The chemistry of photographic sensitizing dyestuffs.** *Chem. and Ind.*, **22,** 351–2. 1949.

Among the dyes used to increase spectral sensitivity of a silver halide emulsion are erythrosin, isocyanine, tricarbocyanines, and azocyanines. It is not the introduction into a sensitizing dye of a nitrogen atom in place of a polymethin group which alters the type of its photographic action, but rather the position occupied by the nitrogen atom. Sensitizing dyes contain neutralized systems in which the anionoid and cationoid centers of the molecule are united directly by a chain of unsaturated units, the induced polarization leading by electrometric displacements to the formation of dipoles.—*R. T. Whittenberger.*

SLOVITER, H. A. **Halogenated benzophenoxazine dyes.** *J. Amer. Chem. Soc.*, **71,** 3360–2. 1949.

The discovery that certain oxazine dyes stain and inhibit the growth of cancer cells stimulated the study of related compounds. Accordingly the halogen derivatives of Nile blue 2B were prepared. No data were given concerning the effect of these derivatives on cancerous cells.—*R. T. Whittenberger.*

ANIMAL MICROTECHNIC

BRECHER, GEORGE. **Phase microscopy in the study of reticulocytes.** *Bul. Intern. Assoc. Med. Museums,* **30,** 99–105. 1949.

Intracellular granules can be identified with phase illumination in unstained rabbit reticulocytes. During reticulocytosis induced in rabbits by phenylhydrazin administration, aggregation of the intracellular granules can be seen in unstained preparations with phase illumination. Examination of stained preparations of rabbit blood after phenylhydrazin administration indicates that the stained reticulum and the granules seen with the phase microscope are closely related but not identical structures.—*Author's summary.*

EDWARDS, J. E., and MALLORY, G. K. **Mallory's phosphotungstic acid hematoxylin stain for alcoholic hyalin.** *Bul. Intern. Assoc. Med. Museums,* **30,** 130–4. 1949.

In 1939 or 1940 the late F. B. Mallory developed a method for staining the alcoholic hyalin of the liver, which he demonstrated in his own laboratory but never published. The method was merely the application of the well-known Mallory phosphotungstic acid hematoxylin technic to alcohol-fixed sections of liver. The present authors, who were associates of his at that time, call attention to the value of the method and propose two modifications. The first modification consists of fixing in 99% isopropyl alcohol (instead of ethyl); this change is optional only, and the authors do not explain what advantage it offers. The second modification consists of mordanting (after removing paraffin from the sections and running through alcohol into water) for 18 hr. in Peer's bichloride mordant at 56°C.; this mordant is a saturated aqueous solution of $HgCl_2$, prepared by adding an excess (about 6.5%) of that salt, keeping it in a paraffin oven and occasionally replacing the water evaporated. The use of this mordant yields sharper staining of nuclei and of the walls of the bile canaliculi.—*H. J. Conn.*

MICROÖRGANISMS

BARTHOLOMEW, J. W., EVANS, E. E., and NIELSON, E. D. **The effect of esterification of protein carboxyl groups on the staining of bacterial cells.** *J. Bact.*, **58,** 347–50. 1949.

Methylation of bacterial cells resulted in reduction or loss of their capacity to stain with basic dyes. The method is as follows: Prepare slides from a distilled water suspension of 24-hr. nutrient agar slant culture; fix with heat; esterify by immersing slides in methyl alcohol containing 0.05 *N* HCl for 7 days; prepare untreated control slides and slides in 0.05 *N* HCl in water; rinse in distilled water; dry; stain with safranin or methylene blue, 0.5% in distilled water buffered at pH 9.0 or 11.5 for 1 min., or with acid fuchsin, 0.5% in solution buffered at pH 2.2, 5.0, or 7.0 for 10 min. Four species of Gram-positive and four of Gram-negative or-

ganisms were studied. Methylation improved staining with acid fuchsin in several of the tests, but was much less consistent in this than in the reduction of the basic staining capacity.—*V. Kavanagh.*

JETER, W. S., and WYNNE, E. S. **Acid fuchsin methylene blue agar: A new differential medium for enteric bacteria.** *J. Bact.*, **58,** 429–32. 1949.

The differential media for Enterobacteriaceae are not always satisfactory because (1) generally only the lactose-fermenting colonies are colored while the nonfermenters are masked by diffusion, (2) the media may be toxic for delicate forms, (3) deterioration may occur with exposure to light, and (4) weak fermenters may not be distinguished from nonfermenters. Eosin and methylene blue (EMB) or eosin and methyl green form dye complexes absorbed only by fermenters. The acid dyes, acid fuchsin, Congo red, and picric acid, and the basic dyes, Bismark brown Y, crystal violet, malachite green, methyl green, methylene blue, nigrosin, thionin, and toluidine blue O (all certified dyes but numbers not given) were tested. Acid fuchsin and methylene blue showed low toxicity, high differential value, and moved independently in all the tests devised. The medium (AFMB) is as follows: Difco peptone, 10 g.; lactose, 10 g.; K_2HPO_4, 3 g.; acid fuchsin, 0.5 g.; methylene blue, 0.05 g.; agar, 15 g.; and distilled water to 1000 ml.; pH, 6.6. The pH can be adjusted by the color of the agar which should be lavender; at pH 7 acid fuchsin decolorizes leaving the agar a clear blue. Lactose-fermenters produce red colonies; nonfermenters, blue. *Proteus* OX19 and *Shigella dysenteriae* and several others grew on AFMB but not on EMB.—*V. Kavanagh.*

PACAUD, ANDRÉ. **Remarques techniques relative à la recherche histochimique de glycogène.** *Bul. Histol. Appl.*, **26,** 153–6. 1949.

The author proposes a modification of the iodine method of demonstrating glycogen histochemically. The reagent used is an iodated gum. To prepare this, a small piece of quite pure gum arabic is suspended in a cloth bag into a flask of double strength Lugol solution (1:2:100), and the flask placed in an incubator at 37–40°C. The gum diffuses and the solution becomes syrupy; on removing to room temperature the syrup gels. This gel is then cut into small pieces, and again suspended in a cloth bag in a solution of double strength Lugol. This process can be repeated several times, each time yielding a gum higher in iodine content; the author finds some materials call for gum which has been iodated in this way through a greater number of stages than do other materials.

The sections of tissue are first stained with Gendre's acetic aniline blue, then are rapidly differentiated in 70% alcohol. Without washing out the alcohol, a drop of the iodated gum is placed on the section, and the alcohol allows it to diffuse rapidly. The section is left exposed to the air but protected from dust, for about ten minutes, when it is covered with a coverslip. Looked at immediately such a preparation is cloudy, but it clarifies slowly with the sublimation of the iodine. A finished preparation shows the placs of glycogen colored a brilliant mahogany on a blue background.—*H. J. Conn.*

SMYTH, J. D. **A quantitative study of the osmium impregnation of the contractile vacuole of *Chilomonas parmecium*.** *Quart. J. Micr. Sci.*, **88,** 65–78. 1947.

Examination of contractile vacuole of *Chilomonas parmecium* during progressive impregnation by Weigl osmic technic revealed that the so-called "osmiophil cortex" appeared first as a number of perivacuolar osmophilic granules. Prolonged impregnation caused these to fuse.

Cultures fixed together and osmicated in separate tubes gave more consistent impregnations in any one series than those both fixed and osmicated separately.

With two samples of OsO_4 used, after normal fixation, A consistently gave impregnations of 62–85% and C impregnations of 25–36%.

Constant mixing during fixation and osmication was not advantageous and gave more irregular results than normal methods.

Time of fixation, 5–90 minutes, had no effect on impregnation.

Irregularities of impregnation were due to the presence of a trace of some retarding agent, possibly introduced at time of fixation.

It was shown that the osmiophily of the perivacuolar region in *Chilomonas* did not vary with the vacuolar phase.

VERNE, JEAN, HÉBERT, SIMONE, and de CHARPAL, O. **La localization histochimique de la phosphatase alcaline dans les dents en évolution chez le rat.** *Bul. Histol. Appl.*, **26,** 170–7. 1949.

Because of the difficulty in decalcifying teeth without destroying phosphatase, and the fact that the calcium phosphates naturally present give a false positive reaction in the Gomori technic, it has been necessary to modify that procedure in studying the localization of phosphatase in developing teeth. The procedure is as follows:

(1) The fragments are fixed in alcohol and passed into distilled water. (2) They are then decalcified for 1–11 days at 10°C. in the following mixture: 70 ml. 10.5% citric acid, 30 ml. N/10 HCl, 0.2 ml. 1% ZnCl, and one drop of chloroform. (The low temperature prolongs the decalcification, but minimizes the destruction of phosphatase.) (3) The fragments are then washed several hours in running water. (4) The enzyme is activated at 37°C. for 3–6 hr. in 1% sodium barbiturate, to which is added 0.075% glycocol. (5) Washing in tap water is carried out for 2–4 hr. (6) Dehydration and embedding in paraffin follows. The reaction for phosphatase is performed according to the Gomori technic.—*H. J. Conn.*

HISTOCHEMISTRY

ARVY, L., and GABE, M. **Mise en évidence simultanée du fer figuré et de la phosphatase alcaline sur coupe à la paraffine.** *Bull. Histol. Appl.*, **26,** 189–91. 1949.

The authors call attention to the fact that the chief inconvenience of the Gomori technic is that the activity of phosphatase is not differentiated from calcium normally in the tissue or from other metals (including iron) which form a black sulfide. They show that in the case of iron this distinction can be brought out by submitting the sections already treated by the Gomori technic to potassium ferricyanide, thus obtaining the intense blue of the Turnbull blue reaction wherever iron is localized. The method is:

Fix in alcohol or acetone; embed in paraffin, timing the steps as required for the demonstration of alkaline phosphatase; section, mount and dry rapidly; incubate in Gomori's medium; rinse rapidly in 1% $Ca(NO_3)_2$; treat with 2% cobalt nitrate; wash thoroughly in distilled water; treat 5 min. in a "commercial solution of $(NH_4)_2SO_4$" diluted with 60 vol. of distilled water; treat 20–30 min. in a mixture of equal parts of 10% potassium ferricyanide and 1% HCl; wash in distilled water, dehydrate, and mount in balsam. By this technic iron deposits appear blue-green, the places of phosphatase activity brown or black.—*H. J. Conn.*

BAKER, J. R. **The histochemical recognition of certain guanidine derivatives.** *Quart. J. Micr. Sci.*, **88,** 115–21. 1947.

Details are given of a method whereby Sakaguchi's test may be applied to paraffin sections. Arginine, being a constituent of most proteins, is by far the commonest positively-reacting substance that occurs in the tissues of plants and animals. Galegine and agmatine, which also react positively, are of very restricted occurence.

LILLIE, R. D., and MOWRY, R. W. **Histochemical studies on absorption of iron by tissue sections.** *Bul. Intern. Assoc. Med. Museums,* **30,** 91–4. 1949.

Selective impregnation of mucins, reticulum and collagen may be achieved on alcohol-fixed tissues by incubation in 1% mannitol, M/100 Fe Cl_3 at pH 5.0, followed by application of acetic acid, potassium ferrocyanide mixture. A similar $FeCl_3$ solution at pH 8.0 impregnated gastric surface mucin of rabbits and guinea pigs and gastric argentaffin cells of rabbits. Formalin fixation decreases or inhibits these actions, except the impregnation of mucins from the pH 5.0 $FeCl_3$ mannitol. —*Author's summary.*

MOWRY, ROBERT W. **A comparison of technical procedures for the histochemical preservation of alkaline phosphatase.** *Bul. Intern. Assoc. Med. Museums,* **30,** 95–8. 1949.

The author investigates several variations of certain preliminary steps (e.g., fixation, clearing, paraffin infiltration) necessary in preparing material for the Gomori method for demonstrating alkaline phosphatase. He emphasizes the impor-

tance of a standard procedure, and recommends the following: Fix in 65% ethyl alcohol for 18–24 hr.; change to 95% alcohol for 6–8 hr.; leave overnight in absolute alcohol; clear in two 30-min. changes of petroleum ether; infiltrate for 30 min. in paraffin at 60°C. in a vacuum. An incubation time of 30–60 min. suffices to demonstrate the enzyme activity in various laboratory animals, but a longer time may be required for some human tissues.—*H. J. Conn.*

ROYER, G. L. **Chemical microscopy in dyeing and finishing.** *Anal. Chem.*, 21, 442–7. 1949.

A modified Hardy microtome is used for cutting fibers. For embedding wool, cotton and viscose rayon fibers, cellulose acetate dissolved in acetone is used. Collodion in ether-alcohol is used with cellulose acetate fibers. Structural characteristics which facilitate the identification of various types of fibers are described.—*R. T. Whittenberger.*